THE UNIVERSITY *of* EDINBURGH

CELLULAR MECHANISMS OF PANCREATIC ENZYME SECRETION

by

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PREFACE

This thesis is divided into four parts. Part I, which forms the major part of the work, is concerned with an investigation of enzyme secretion from an isolated preparation of the pancreas together with the effects of changes in the extracellular ionic environment and certain drugs on this process. In parts II, III and IV the results of preliminary experiments on isolated pancreatic cells, their growth and electrophysiological characteristics in culture are presented.

It is a pleasure to express my sincere thanks to Professor T. Scratcherd, who acted as my supervisor and to Dr. R.M. Case. I consider it a privilege to have worked with them. My thanks are also due to a number of colleagues who kindly gave me their time during the course of this work. Dr. T. Elsdale for his advice on cell culture; Dr. M.O. Wright for help with the electrophysiological studies; Mr. N. Davidson for advice on photographic matters and Dr. J.R. Greenwell for help with computing and statistics. Lastly I am indebted to Mrs. Thea Saunders and her staff at the Teaching and Research Centre, Western General Hospital for providing excellent technical assistance and to the Faculty of Medicine for financial support.

Some of the work presented in this thesis has been published, as detailed below.

B.E. ARGENT

JUNE 1975.

Publications

ARGENT, B.E., CASE, R.M., POOLE, L.M., SCRATCHERD, T. (1970). Stimulation of enzyme secretion from the perfused cat pancreas by potassium. J. Physiol. 208, , 79-80 P.

ARGENT, B.E., CASE, R.M. & SCRATCHERD, T. (1971). Stimulation of amylase secretion from the perfused cat pancreas by potassium and other alkali metal ions. J. Physiol. 216, 611-624.

ARGENT, B.E., CASE, R.M., FRASER, M.P. & SCRATCHERD, T. (1972). The effects of calcium on volume and amylase secretion from the perfused cat pancrease. J. Physiol. 224 29-30 P

ARGENT, B.E., CASE, R.M. & SCRATCHERD, T. (1973). Amylase secretion by the perfused cat pancrease in relation to the secretion of calcium and other electrolytes and as influenced by the external ionic environment. J. Physiol. 230 575-593.

CONTENTS

Abbreviations

Standard International Unit equivalents are shown in parenthesis.

ACh.....Acetylcholine.

ACTH.....Adrenocorticotrophic Hormone.

ATP.....Adenosine Triphosphate.

Approx.....Approximately.

°C.....Degrees Celsius.

CCK-PZ or PZ-CCK.....Cholecystokinin-Pancreozymin.

Cells 3T3, BICR, MIRK, CHO, KB, L ..... Cell lines established in culture. For detailed imformation on the nomenclature of established cell lines see PAUL (1970).

C.H.R. Units... Crick, Harper, Raper units of CCK-PZ activity.

Ci.....Curie.

Ci/mMol.....Curies per millimole.

μCi/ml.....microcuries per millilitre. (microcuries per cubic centimetre).

cms.H_2O.....Centimetres of water. (98.0665 Newtons per square metre)

DNA.....Deoxyribonucleic acid.

dpm.....Disintegrations per minute.

EDTA.....Ethylenediaminetetra-acetic acid.

EGTA.....Ethylene-glycol-bis(β aminoethyl ether)-N-N^1 tetra-acetic acid.

EM.....Electronmicroscope.

g.....Gramme.

ng.....Nanogramme.

I.U.....International Unit of enzyme activity.

K_{out}.....Extracellular potassium concentration.

l .....Litre (cubic decimetre).

L.H.....Luteinizing hormone.

log.....Logarithm.

M.....Molarity. 1 Molar= molecular weight in grammes per litre.

meq or m equiv.....Milliequivalent. $1\,meq = \frac{\text{atomic weight}}{\text{valency}} \cdot \frac{1}{1000}$ grammes.

mEq/l.....Milliequivalents per litre. (milliequivalents per cubic decimetre)

mg.....milligramme.

mg/kg.....milligrammes per kilogramme.

mg%.....mg per 100 millilitres. (milligrammes per 100 cubic centimetres).

mM.....millimolar.

mV.....millivolt.

MΩ.....Mega Ohm.

N.....Normality. 1N=1equivalent in grammes per litre.

Na/K ATPase....Sodium potassium activated magnesium dependent adenosine triphosphatase.

pH.....$-\log_{10}$ [hydrogen ion concentration].(nanogrammes hydrogen ions per cubic decimetre).

pK^+.....Potassium ion permeability.

pNa^+.....Sodium ion permeability.

PVG(strain)..... Piebald Virol Glaxo.

TCA.....Trichloroacetic acid.

Thyroid Hormones..
- MIT...Monoiodotyrosine.
- DIT...Diiodotyrosine.
- T_3 ...Triiodothyronine.
- T_4....Thyroxine.

TPBR.....Total protein bound radioactivity.

µ.....micron. (micrometre).

µg.....microgramme.

cpm.....Counts per minute.

Chemicals and Culture Media

(1) Inorganic Chemicals.

Rubidium chloride.
Calcium chloride. Analar.
Caesium chloride. Analar.
Lithium chloride.
Magnesium chloride. Analar.
Potassium chloride. Analar.
Potassium hydrogen carbonate. Analar.
Sodium Hydrogen carbonate. Analar.
Sodium chloride. Analar.
Sodium dihydrogen orthophosphate. Analar.

All inorganic chemicals were supplied by BDH Chemicals Ltd.

(2) Organic Chemicals.

Acetylcholine chloride.	BDH
Atropine sulphate.	BDH
Bovine serum albumin (crystallized).	Sigma
Carbamylcholine chloride.	BDH
Colchicine.	BDH
Ethylenediaminetetra-acetic acid. disodium salt. Analar.	BDH
Ethylene-glycol-bis(β aminoethyl ether) -N-N'tetra- acetic acid.	Sigma
Eosin Y.	BDH
Eserine sulphate.	BDH
Glucose. Analar.	BDH
Hyoscine hydrobromide.	BDH
Nembutal (sodium pentobarbitone 60mg/ml)	Abbot Laboratories.
Noradrenaline.	BDH
Nystatin.	E.R.Squibb & Sons.
Phenoxybenzamine hydrochloride.	Smith, Kline & French.
Sucrose. Analar.	BDH
Tetracaine.(Amethocaine).	Sigma
Trichloroacetic acid. Analar.	BDH

(3) Enzymes.

Collagenase. Form 1. ex Clostridium histolyticum.	Koch Light.
Hyaluronidase. ex bovine testis.	Koch Light.
Pancreatin. ex Pig pancreas	BDH.
Pronase. ex Streptomyces griseus	Koch Light.
Trypsin. ex Beef pancreas. Crystalline	BDH.

(4) Protein Hormones.

Crude secretin and cholecystokinin-pancreozymin were prepared by the method of CRICK, HARPER & RAPER (1949). Pure secretin and cholecystokinin-pancreozymin were gifts from Professor V. Mutt, Karolinska Institutet, Stockholm.

(5) Radioactive chemicals.

L-Leucine-4-5-H3. Specific activity approximately 50 Ci/mMol. Radiochemical Centre, Amersham.

(6) Detergents.

Decon 75 Decon Laboratories Ltd. Brighton.

(7) Culture media.

All items listed in this section were purchased from Biocult Laboratories LtD, Paisley, Scotland.

Hanks balanced salt solution (Hanks BSS).

HANKS, J.H. & WALLACE, R.E. Proc. Soc. Exp. Biol. Med. 71, 196-200 (1949).

Eagles minimum essential medium (Eagles MEM).

EAGLE, H. Science, 130, 432 (1959).

Ham's F12 medium.

HAM, R.G. Proc. Nat. Acad. Sci. (Wash). 53, 288 (1965).

McCoy's 5A medium.

McCOY, T.A., MAXWELL, M. & KRUSE, P.F. Proc. Soc. Exp. Biol. Med. 100, 115 (1959).

NCTC 109 medium.

EVANS, V.J., BRYANT, J.C., KERR, H.A. & SCHILLING, F. L. Exp. Cell. Res. 36, 439 (1964).

Calf serum.

SUMMARY

PART I

1. Amylase secretion from the perfused cat pancreas consists of two components: a small continuous basal secretion and a stimulated secretion in response to acetylcholine or cholecystokinin-pancreozymin. The response to small doses of either stimulant was repeatable over several hours.

2. The calcium concentration of pancreatic juice, always less than that of the perfusate, was normally constant above secretory rates of 0.15 g/10 min. However, when the concentration of enzymes in the juice rose, either after stimulation or at very low secretory rates, the calcium concentration rose in parallel, suggesting that this calcium is bound to, or is a component of pancreatic enzymes.

3. Elevation of the perfusate calcium concentration resulted in a parallel increase in the calcium concentration of the pancreatic juice.

4. Calcium-free solutions initially caused a small reduction in basal and stimulated amylase secretion and, after prolonged periods of perfusion, abolished stimulated secretion and caused a reduction in electrolyte secretion. The latter was completely reversed by calcium-rich perfusates but the effects on enzyme secretion were only partially reversible.

5. Calcium-rich perfusates had no effect on the rates of electrolyte secretion but potentiated submaximally stimulated amylase

secretion.

6. Barium did not substitute for calcium in supporting pancreatic secretion.

7. Alterations in the extracellular concentrations of sodium, and magnesium had no effect on amylase secretion.

8. It is concluded (a) that calcium is secreted into the pancreatic juice in two fractions, one associated with enzymes and the other with the electrolyte component of the juice ; and (b) that calcium ions play an important role in the stimulus-secretion coupling of pancreatic acinar cells, but that the effects of calcium depletion on electrolyte secretion may principally be due to alterations in the permeability of the duct system.

9. Increasing the perfusate potassium concentration (at the expense of sodium ions) caused a copious secretion of amylase from the gland, reduced the volume rate of secretion and caused vaso-constriction.

10. Rubidium and caesium had similar effects to potassium: lithium, though depressing secretory rate, had no effect on enzyme secretion or vasoconstrictor action.

11. Amylase secretion was detected at potassium concentrations of 30 mM and was maximal at 80-90 mM, output declining as the concentration was raised to 120 mM.

12. Amylase secretion was maximal during the first few minutes of exposure to excess potassium, but remained above basal levels throughout the test period. Secretory rate was depressed by a constant amount during the test period.

13. Atropine sulphate blocked the effect on enzyme secretion without affecting the reduction in secretory rate.

14. During perfusion with excess potassium a vasodepressor material with the properties of acetylcholine was detected in the

effluent from the gland.

15. The reduction in secretory rate, when perfusate sodium was replaced by potassium, was equal to that obtained when sodium was replaced by sucrose.

16. It is concluded that potassium stimulates amylase secretion indirectly by releasing acetylcholine from nerve terminals in the gland, and that the reduction in secretory rate is due not to excess potassium but to sodium deficiency.

17. The local anaesthetic tetracaine inhibited amylase secretion at a lower concentration than that required to inhibit electrolyte secretion.

18. The antimitotic drug cholchicine caused a progressive increase in the rate of electrolyte secretion stimulated by supra-maximal secretin infusion. In one experiment it also blocked stimulated amylase secretion.

19. The polyene antibiotic nystatin inhibited electrolyte secretion and stimulated enzyme secretion.

PART II

1. Digestion of the guinea pig pancreas with collagenase and pronase yielded cell suspensions consisting of 90-95% acinar cells.

2. The yield of isolated cells was approximately 5-10% on a wet weight basis.

3. The acinar cells were rounded up but maintained their *in situ* polarity of apical zymogen granules and basal nuclei.

4. On the basis of a dye exclusion test the isolated cells were 90-95% viable.

5. The isolated acinar cells incorporated a radioactive amino-acid into cellular proteins at a linear rate for up to 5 hours after isolation.

6. Pulse labelling experiments demonstrated that the acinar cells were capable of secreting *de novo* synthesised protein in response to CCK-Pz but not to cholinergic stimuli.

7. It is concluded that a specific inactivation of the muscarinic acetylcholine receptor on the acinar cells has occurred, and that this is probably due to the use of pronase in the isolation technique.

PART III

1. A technique has been developed for the primary culture of suspension of isolated guinea pig pancreatic cells.

2. The cells grow best in a medium consisting of McCoy's 5A + 20% calf serum.

3. Both epithelial and fibroblastic cell morphologies were present in the cultures. The percentage of fibroblastic cells increased with age of the culture.

4. Attempts at cloning the epithelial cells were unsuccessful.

5. Amylase was not detectable in the cultures.

6. In agreement with previous observations by other workers it is concluded that the acinar cells undergo dedifferentiation in culture. Their descendents were present as unspecialised epithelial cells.

PART IV

1. The membrane potentials of epithelial cells in primary cultures derived from the guinea pig pancreas have been determined.

2. The mean resting potential was 7.4 $\pm$ 0.2 mV (inside negative).

3. The frequency distribution for the resting potentials indicated that one population of cells was being sampled.

4. Increases in extracellular K^+ caused a fall in the mean membrane potential.

5. Stimulants of pancreatic enzyme secretion, CCK-Pz and carbamylcholine, depolarised the cells whereas secretin had no effect.

6. It is concluded that the dedifferentiated descendents of the acinar cells retain at least one component of their *in situ* physiological response to stimulation.

PART I

PANCREATIC PERFUSION STUDIES

INTRODUCTION

The discovery by JOHN GEORGE WIRSUNG (1643) of the pancreatic duct may be described as the point at which the study of pancreatic physiology began. He recognised that this structure was neither an artery nor a vein since it never contained blood but always a colourless fluid. The first comprehensive investigation into the properties of pancreatic juice was made by de GRAAF (1664). He was the first to successfully cannulate the duct, previously described by WIRSUNG and succeeded in obtaining an ounce of juice from one animal in 7-8 hours. Using a similar technique he was able to collect saliva and pancreatic juice from the same animal and observed, relying mainly on his sense of taste, that the two differed in composition. He considered that the function of pancreatic juice was to react with bile and thus aid in the process of fermentation.

With these discoveries interest in pancreatic physiology faded and it was not until one and a half centuries later that the revival began. In 1823, The Royal Academy of Paris proposed 'Digestion' as the subject of a prize dissertation, and was thus responsible for two notable essays which the judges found impossible to distinguish between. TIEDEMANN and GMELIN (1826), in their dissertation on digestion in the mammal, described the composition of both saliva and pancreatic juice and showed that the latter contained twice as much solid material. They stated that pancreatic juice was neutral or acid, though it became slightly alkaline on standing. LEURET AND LASSAIGNE (1825) were the authors of the other prize winning essay and they paid more attention to the pancreatic juice. They maintained that it was more like saliva than did TIEDEMANN and GMELIN, stating it to be 99% water

and realised that it was definitely alkaline. Their main contribution that acid placed in the duodenum provoked a flow of pancreatic juice and bile was forgotten and it was not until 1893 in PAVLOV'S laboratory that this effect was rediscovered. CLAUDE BERNARD (1849, 1856) was apparently unaware of the work of LEURET and LASSAIGNE and developed a method for stimulating pancreatic juice secretion by the introduction of ether into the duodenum. In his first work (1849) he describes two types of pancreatic juice, that collected immediately after stimulation contained a large amount of active, coaguable material (normal juice) while that collected later contained little (morbid juice). This was the first recognition of the enzyme 'washout phenomenon'. Otherwise the fluids were the same, exhibiting a clearly alkaline reaction. He further ascribed the property of fat emulsification to 'normal' pancreatic juice and more important noted its ability to decompose fat into fatty acids and glycerol. Subsequently (1856) he described the action of pancreatic juice on fats, starch and proteins. At the same time as BERNARD was making his pioneering observations, LUDWIG (1851) showed that secretion of saliva from the submaxillary gland did not consist of a mere filtration of salts and water from the blood through a glandular epithelium, since the pressure developed in the occluded duct of this gland during secretion may become greater than the blood pressure in the carotid artery.

HEIDENHAIN (1868), working with the salivary glands, was the first to discern the dual nature of the secretory processes in digestive glands. These he defined as the transfer of water and crystalloids through the glandular epithelium to the alimentary canal, possibly with the products of cell synthesis e.g. HCl or HCO_3^-, and

the liberation by the glandular cells of the organic colloidal compounds which they have elaborated e.g. enzymes. HEIDENHAIN (1875) also made observations on the histology of the pancreatic gland, describing an inner granulated and outer clear zone in the secreting cells of the fasting dog's pancreas. He also noted that the granulated zone became narrower during food digestion and on occasions disappeared altogether. HEIDENHAIN proposed the name zymogen for that structure within the pancreas which gives rise to the ferments and suggested that the pancreas was not only involved in secreting zymogen granules but also in their laborious manufacture. Confirmation of this view came from KUHNE and LEA (1882) while studying the pancreas of small live rabbits under the microscope. Granules moved from the nuclear zone to the apex of the secretory cells where they diminished in size. Much later, COVELL (1928) reported the same phenomenon.

Towards the end of the nineteenth century the control of pancreatic secretion was also receiving considerable attention. The nervous control of the gland was described in detail by PAVLOV (1910). Stimulating the peripheral end of the cervical vagus caused a free flow of pancreatic juice. When, in PAVLOV's laboratory, DOLINSKY (1894) confirmed the finding of LEURET and LASSAIGNE, that acid, particularly hydrochloric acid, was a good stimulant of pancreatic secretion, PAVLOV repeated his earlier experiments after occluding the pyloric canal. This resulted in the secretion of a viscid juice rich in enzymes. So convinced were the Russian physiologists of the complete control of digestive glands by nerves, that the effects of acid in the duodenum were explained by local nervous reflexes between intestine and pancreas, despite the fact that POPIELSKI (1901) had

excluded all nervous pathways. It was left to BAYLISS and STARLING (1902) to discover that an extract of intestinal mucosa when injected intravenously resulted in a flow of juice from the pancreas. The active principle, designated secretin, was the first of the chemical messengers to be called hormones. This discovery introduced a new concept of the mechanisms by which the secretory activity of digestive glands was regulated.

The enthusiasm of the discoverers of secretin and their supporters was so great however that they denied the secretory action of the vagus nerve on the pancreas, attributing the effects observed by the Pavlovian school to the passage of acid from the stomach into the duodenum. However their observation that stimulation of the nerves to the pancreas, after pyloric occlusion, resulted in the secretion of a juice of low volume but high enzyme content was confirmed by ANREP (1916) and by BABKIN (1924). A year after BABKIN published his results MELLANBY (1925) reported the preparation of an extract from the mucosa of the small intestine, which when infused into cats, caused a constant secretion of water and bicarbonate but a diminished output of enzymes. Since vagal stimulation still resulted in enzyme secretion under these conditions he concluded a dual mechanism for the control of pancreatic secretion, the control of enzyme content being determined by the activity of the vagus nerve, whereas the volume of the bicarbonate solution in which these enzymes were contained is determined by secretin.

This concept held until 1941 when HARPER and VASS observed that the enzyme secretion stimulated by the presence of food in the small intestine of cats was not abolished by denervation of this section of the gut. Having confirmed that this effect was not due to the

release of secretin these workers concluded that their results could only be explained by the presence of intrinsic nerve plexuses in the intestine or some humoral mechanism other than secretin. HARPER and RAPER (1943) later succeeded in preparing an extract of small intestine having a marked effect on pancreatic enzyme secretion but no effect on the volume flow of pancreatic juice. They called the active principle of this extract pancreozymin.

Pure preparations of secretin and pancreozymin have now been obtained (MUTT and JORPES, 1967) and secretin has been synthesised (BODANSZKY, 1973).

Both these hormones are polypeptides with different amino-acid compositions. Secretin has a molecular weight of approximately 3,500 and consists of 27 aminoacids. The molecule of pancreozymin is slightly larger consisting of 33 aminoacids. Unlike secretin which requires a complete molecular structure for biological activity the full spectrum of activities for pancreozymin can be obtained with the 8 C-terminal amino-acids (JORPES, 1968). The 5 C-terminal amino-acids of pancreozymin are identical with those of gastrin (GREGORY, HARDY, JONES, KENNER and SHEPPARD, 1964).

In addition to stimulating pancreatic enzyme secretion pancreozymin also possesses a gall bladder contracting activity (DUNCAN, HARPER, HOWAT, OLEESKY and VARLEY, 1950). A molecule possessing a similar activity on the toad gall bladder, called cholecystokinin, had previously been isolated by IVY and OLDBERG (1928) from extracts of upper intestine mucosa. It was later recognised that these extracts contained a pancreozymin activity. In the course of their attempts to purify cholecystokinin, JORPES and his colleagues noted that the pancreozymin activity of the extracts

also increased. For this reason they employ the name cholecystokinin-pancreozymin (CCK-PZ) to describe the active material. It is now evident that these activities are both shared by the same molecule (JORPES, 1968).

On the basis of morphological evidence the site of origin of the enzyme component of pancreatic juice has been established as the acinar cells. These cells are characterized by the presence of abundant rough surfaced endoplasmic reticulum and large zymogen granules in their cytoplasm. Enzyme is released from these cells in a small volume of fluid whose composition is very similar to that of intracellular fluid (CASE, HARPER and SCRATCHERD, 1969a).

The site of water and electrolyte secretion has been identified less easily. Pathological and physiological evidence (GROSSMAN and IVY, 1946; de ALMEIDA and GROSSMAN, 1952; KALSER and GROSSMAN, 1954; DREILING, JANOWITZ and HALPERN, 1955; BECKER, 1962) have indicated that the centroacinar and intercalated duct cells are primarily involved. However these cells neither possess the ultrastructural features, i.e. high mitochondrial density, that characterize electrolyte and water secreting cells in other tissues (EKHOLM, ZELANDER and EDLUND, 1962) nor undergo significant morphological changes following stimulation by secretin *in vitro* (ICHIKAWA, 1965) or *in vivo* (RIBET, FEDOU and FREXINOS, 1969).

The essential features of the synthesis, intracellular transport, and storage of digestive enzymes within the pancreatic acinar cell are well defined. After synthesis on the ribosomes of the rough-surfaced endoplasmic reticulum, the digestive enzymes, or their zymogens, are transferred via the cisternae of the rough-surfaced endoplasmic reticulum and the small vesicles of the peripheral Golgi

complex to the condensing vacuoles, which are subsequently transformed into zymogen granules by progressive filling and concentration of their contents and stored in the apical region of the cell. Morphological studies have shown that, following stimulation, zymogen discharge (which I shall refer to as secretion) involves the movement of the granule to the cell surface, where its membrane fuses with the plasma membrane thus extruding its contents by exocytosis into the acinar lumen (for review see SCHRAMM, 1967). However, little is known of the mechanical processes involved in movement of the zymogen granules, or how they are controlled (stimulus-secretion coupling).

The first part of this thesis explores the influence of extracellular ionic composition on these processes in the perfused cat pancreas. Such an analysis in the intact gland is complicated by the need for a background secretion of electrolytes and water (to act as a vehicle for the enzymes), which itself is markedly influenced by the composition of the perfusing fluid (CASE, HARPER and SCRATCHERD, 1968, 1969b). Nevertheless a perfused preparation does offer considerable advantages over alternative <u>in vitro</u> techniques, notably rapid and reversible alteration of the extracellular fluid composition, efficient oxygenation and collection of uncontaminated secretory products. In addition, the simultaneous measurement of electrolyte secretion has allowed observations to be made on this component of the pancreatic juice.

METHODS

A saline-perfused preparation of the cat's pancreas (CASE, et al., 1968) was used in all experiments. Cats of either sex weighing 0.4-4.2 kg were denied food for 18 hr. before the experiment. Anaesthesia was induced and maintained by Nembutal (sodium pentobarbitone; 60 mg/kg) administered intraperitoneally. The circulation through the isolated pancreas was maintained by pumping a balanced salt solution through the coeliac and superior mesenteric arteries and collecting the effluent via a retrograde catheter in the superior mesenteric vein (Fig. I). To isolate the pancreas, the inferior mesenteric artery and vein were ligated and the superior mesenteric artery and vein tied distal to the inferior pancreaticoduodenal artery, which supplies part of the duodenum and pancreas. This devascularised the gut and facilitated its removal beyond the portion of the duodenum related to the head of the pancreas. Similarly the mesentery and spleen were carefully removed after section of their vascular supplies. The blood supply to the stomach was cut off, leaving intact the superior pancreatico-duodenal vessel supplying the pancreas and the duodenum. The stomach was removed. Next the small vessels between the duodenum and the head of the pancreas were ligated. The pancreatic duct was cannulated using a narrow bore, stainless steel cannula. A length of polyethylene tubing of the widest bore practicable (approx. 2.5 mm) was filled with heparinised saline and used to cannulate the aorta in the direction of the heart. It was tied in place with its tip just distal to the opening of the superior mesenteric artery. The lumbar arteries leaving the aorta between the tip of the tube and the coeliac axis were ligated. The

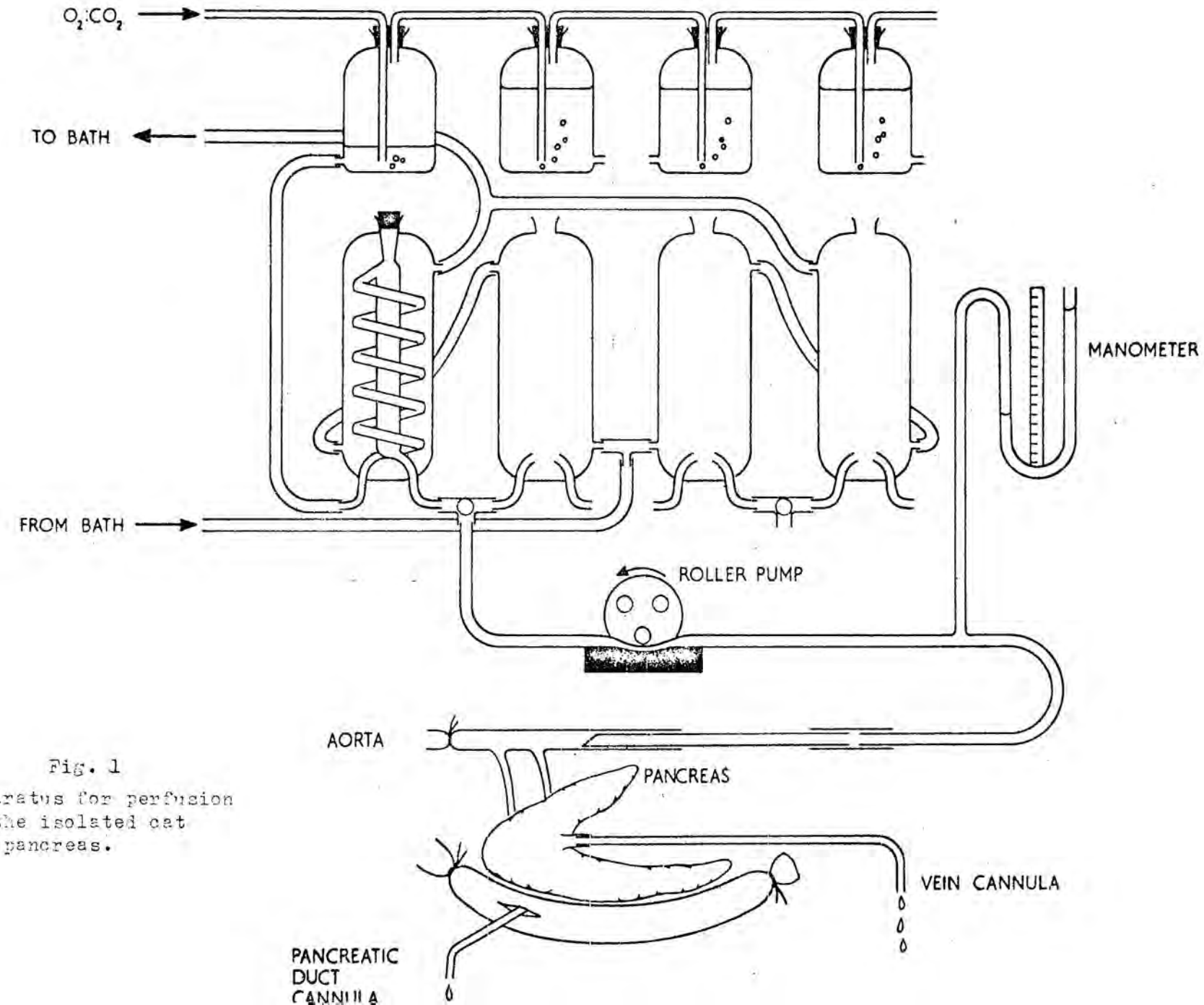


Fig. 1
Apparatus for perfusion of the isolated cat pancreas.

superior mesenteric vein was cannulated, the aorta ligated above the coeliac axis and the portal tract tied thus completing the perfusion circuit. After perfusion was established, the animal was killed by bleeding but for convenience the pancreas was left in situ.

The standard perfusion fluid, isosmolal with cat's plasma, had the following composition in mM NaCl 125, KCl 4.3, $NaHCO_3$ 25 $MgCl_2$ 0.5, NaH_2PO_4 1.0, $CaCl_2$ 1.25 and glucose 5. In some experiments the $MgCl_2$ and $CaCl_2$ concentrations were 1.0 and 2.5 mM respectively. When the perfusate concentration of potassium, calcium and magnesium were altered, or when rubidium, caesium and lithium were added (as their chlorides), isosmolality was maintained by omission of appropriate amounts of sodium chloride. When sodium was removed an osmotically equivalent amount of sucrose was added. The fluids were filtered through Whatman No. 2 paper before use and gassed continuously with oxygen 95% and carbon dioxide 5%. The final pH of the perfusion fluid was 7.4. From a reservoir the fluid entered a heat-exchange coil maintained at a temperature of 38°C and was infused by means of a roller pump into the gland's arterial supply at a rate of 4-6 mls/min and a pressure of about 30-75 cms H_2O. A bank of four reservoirs allowed rapid changes in the composition of the perfusion fluid to be made.

In all experiments electrolyte secretion was stimulated by infusing secretin into the arterial cannula using a motor driven syringe. Enzyme secretion was stimulated by rapid pulses or prolonged infusions of acetylcholine chloride or cholecystokinin-pancreozymin (CCK-P%). Secretin and CCK-P% were prepared by the method of CRICK, HARPER and RAPER (1949), though in some experiments purer preparations

(G.I.H. Laboratory, Karolinska Institutet, Stockholm) were used.

Pancreatic juice samples were collected in plastic tubes and weighed. Either or both of two indices of enzyme secretion were measured: total protein (mg) estimated by the method of LOWRY, ROSENBROUGH, FARR and RANDALL (1951), and amylase activity (i.u.) estimated by the Nørby method (LAGERLOF, 1942). The concentrations of calcium and magnesium in pancreatic juice and perfusion fluids were estimated by atomic absorption spectrometry (Unicam SP90) and sodium and potassium by flame photometry (Mark II, Evans Electroselenium Ltd.). The osmolalities of the perfusion fluids were determined using an Osmet Precision Osmometer (Precision Systems Ltd.) or a Fiske Osmometer (Model G62, Fiske Associates Inc.).

In some experiments the effluent from the pancreas was assayed for acetylcholine. The arterial blood pressure of the eserinized eviscerated cat was used for this assay because in this method the interference by potassium is least and in a direction opposite to the effect of acetylcholine. (see BROWN and FELDBERG 1936). In addition, since isolation of the pancreas incidentally results in an eviscerated cat preparation, it was possible to perform both perfusion and assay experiments concurrently in the same animal. In these experiments therefore the aorta was not cannulated. Instead, the superior mesenteric artery was ligated and the perfusion fluid led directly into the coeliac axis. This procedure had no detectable effect on the functioning of the gland. In all assay experiments although the circulation through the perfused pancreas and the remainder of the cat appeared not to mix the coeliac ganglia were ablated to eliminate the possibility of acetylcholine being released from these structures. Samples of effluent were injected into the

cat's circulation via a polyethylene catheter in either the external jugular or saphenous vein. To prevent hydrolysis of acetylcholine, eserine sulphate was added both to the perfusion fluids (10 mg/l) and to the cat's circulation (0.15 mg/kg). Mean arterial blood pressure was measured by means of a Kistler type 412 transducer connected to a polyethylene catheter in the carotid artery and recorded after amplification (Kistler Charge Amplifier Type 566) on a Servoscribe potentiometric recorder (Type RE.511.20).

In another group of experiments the effects of high potassium perfusion fluids and noradrenaline were tested on the perfusion pressure and rate of perfusate flow through the gland. Perfusion pressure was measured using a medical transducer type 4/88 (S.E. laboratories Ltd.). and perfusate flow rate using a Devices impulse integrator (type 3210). Both records were displayed on a servoscribe potentiometric recorder (Type RE.511.20). Where statistical analysis has been employed results are expressed as the mean $\pm$ standard error of mean (number of observations).

RESULTS

A. NORMAL PERFUSATE

(1) Enzyme Secretion by the perfused cat pancreas: There was usually no measurable secretion from the unstimulated saline-perfused cat's pancreas. In the absence of electrolyte secretion, CCK-P% and acetylcholine were unable to evoke any detectable amylase secretion. During secretin-stimulated electrolyte secretion amylase secretion consisted of two components: a small continuous basal secretion and a stimulated secretion which was dose dependent and occurred in response to exogenously administered acetylcholine or CCK-P%. The rate of basal amylase secretion varied in different animals. Control experiments indicated that a fall in basal amylase secretion normally occurred during the course of an experiment (Fig. 2). If the perfused gland was stimulated to secrete large amounts of enzyme the response to the same dose of stimulant decreased during the course of an experiment. With small doses the secretory response was repeatable over several hours. (Fig. 2). The response to the first dose of enzyme stimulant was often greater than subsequent responses and has therefore been ignored in all experiments.

The stimulation of enzyme secretion by acetylcholine and CCK-PZ was an evanescent process. Fig. 3 shows that in response to a single dose of acetylcholine the peak concentration of amylase in the juice is reached after 3 minutes and the response is essentially completed within 5 minutes.

(2) The calcium concentration in pancreatic juice: The concentration of calcium in pancreatic juice collected from glands stimulated maximally by secretin was always less than that of the perfusion fluid. In eleven experiments in which the gland was perfused with normal perfusate (2.82 ± 0.09 m-equiv Ca/l) the mean concentration of calcium in the pancreatic juice was 0.63 ± 0.04 m-equiv/l. (78). At secretory rates between 0.15 g/10 min and 0.85 g/10 min the concentration of calcium was independent of flow rate (Fig. 4). In one experiment where the gland was stimulated to secrete at flow rates below 0.15 g/10 min the concentration of calcium in the juice increased with decreasing flow rates as did the concentration of amylase (Fig. 5).

Following stimulation with acetylcholine the output of calcium in the pancreatic juice increased in proportion to the total amount of amylase secreted (Fig. 6). This increased output of calcium paralleled the increased output of amylase when the stimulant was administered as a pulse or as an infusion (Fig. 7)

B. Alterations in perfusate Ca concentration

(1) The effects of Ca-free perfusates on electrolyte and enzyme secretion: In nine experiments prolonged perfusion with calcium-free solutions containing the specific calcium chelator ethylene-glycol-bis (β aminoethyl ether)-N-N^1 tetra-acetic acid (EGTA) 10^{-5}M caused a progressive inhibition of electrolyte secretion which became apparent after 50-70 min. (Figs. 8 and 9). In the early stages this inhibition could be reversed by a return to normal perfusate but when

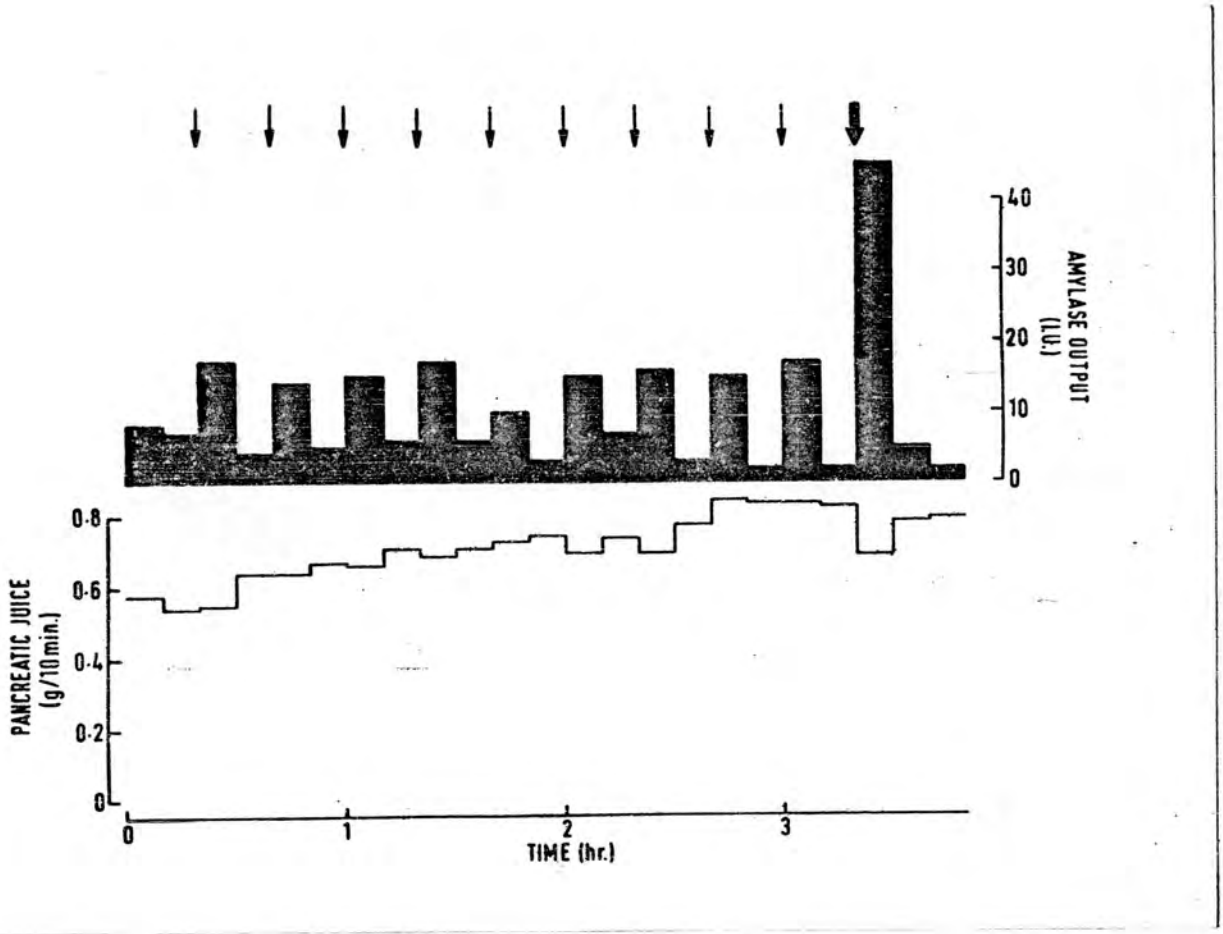


FIG. 2

The secretion of amylase by the perfused cat pancreas. Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supra-maximal dose (30 µg/min). The thin arrows represent single injections of 0.1 mg of CCK-PZ prepared by the method of CRICK, HARPER and RAPER (1949). The thick arrow represents a larger dose (1.0 mg) of the same stimulant.

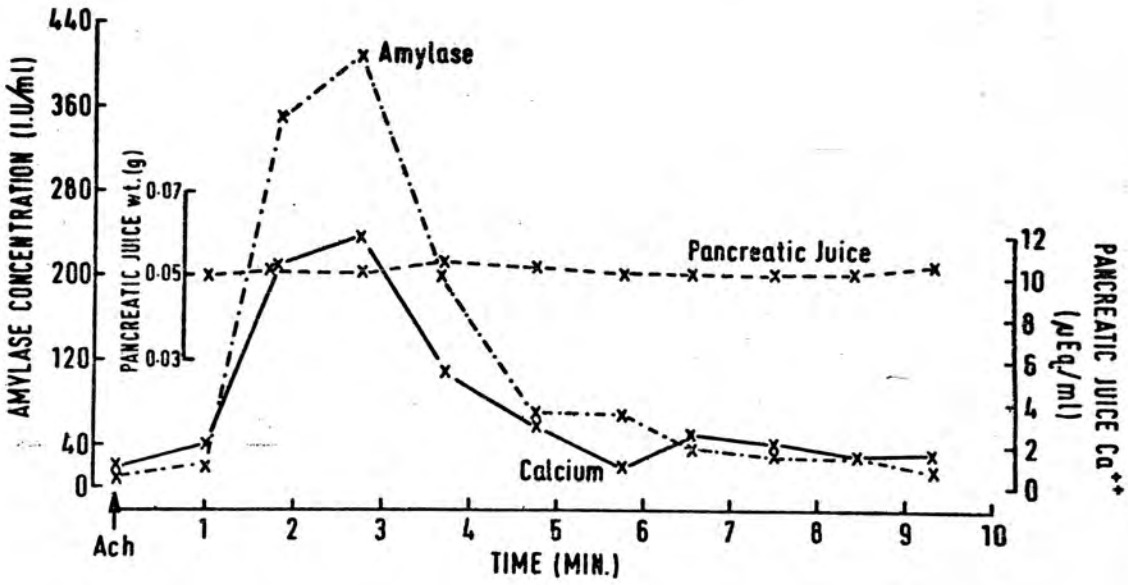


FIG. 3

The time course of amylase secretion from the perfused cat pancrease in response to a single dose of acetylcholine (Ach). Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supramaximal dose (30 μg/min). At zero time (arrow) a single large dose of ACh (1 μg) was rapidly injected into the gland's arterial supply.

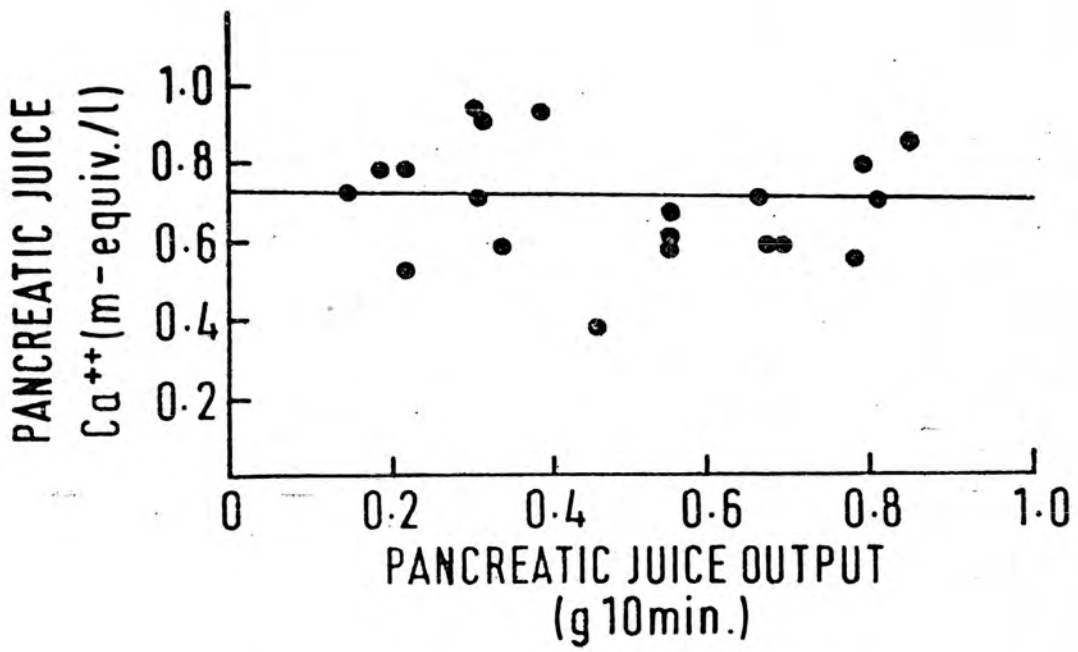


FIG. 4

The secretion of calcium in pancreatic juice. One experiment in which the rate of electrolyte secretion was varied by changing the rate at which secretin was infused into the gland. The solid line is a calculated regression line the slope of which is not significantly different from 0 ($P > 0.5$).

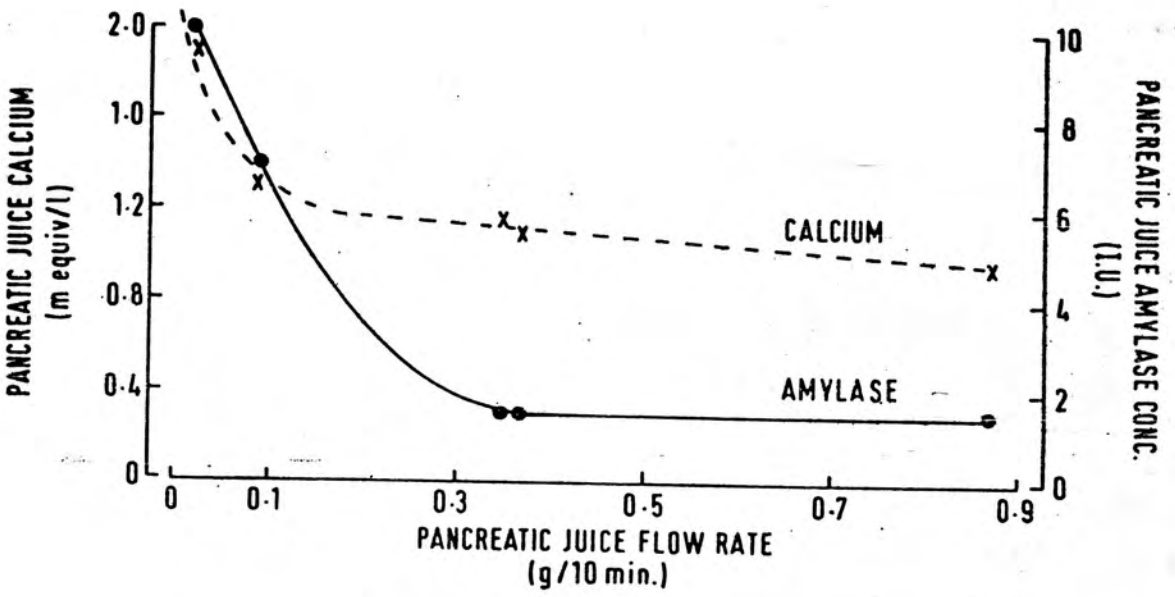


FIG. 5

The calcium and amylase content of pancreatic juice. One experiment in which the rate of electrolyte secretion was allowed to fall to very low levels by reducing the rate of secretin infusion. The perfusate calcium concentration was 2.5 mM.

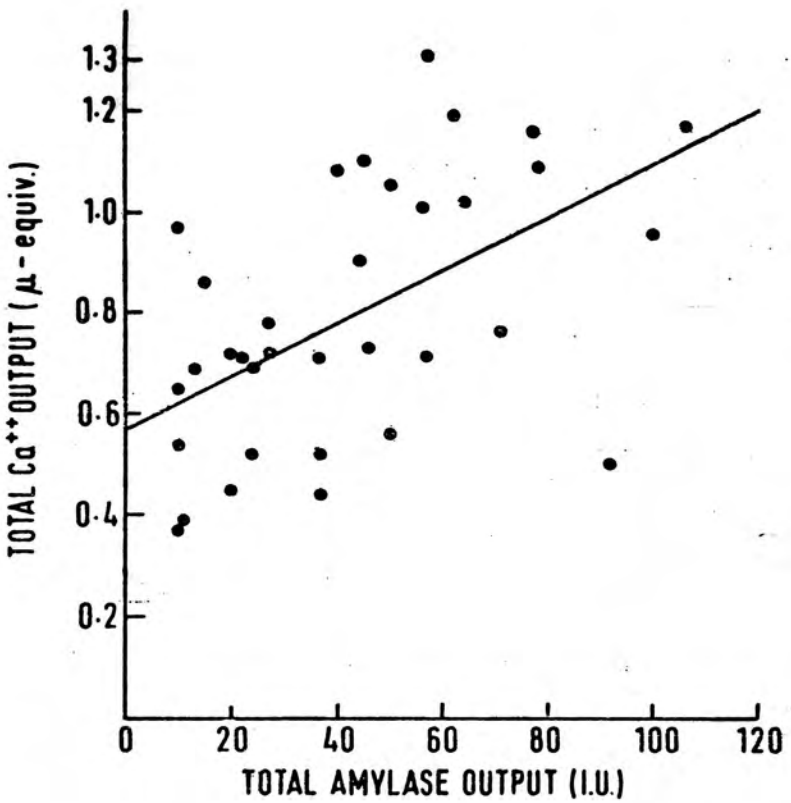


FIG. 6

The secretion of calcium and amylase in pancreatic juice. The points represent a total of thirty-five observations from twelve experiments in which glands were stimulated to secrete amylase by various single doses of acetylcholine (25 ng - 1 μg). The solid line is a calculated regression line ($P < 0.001$).

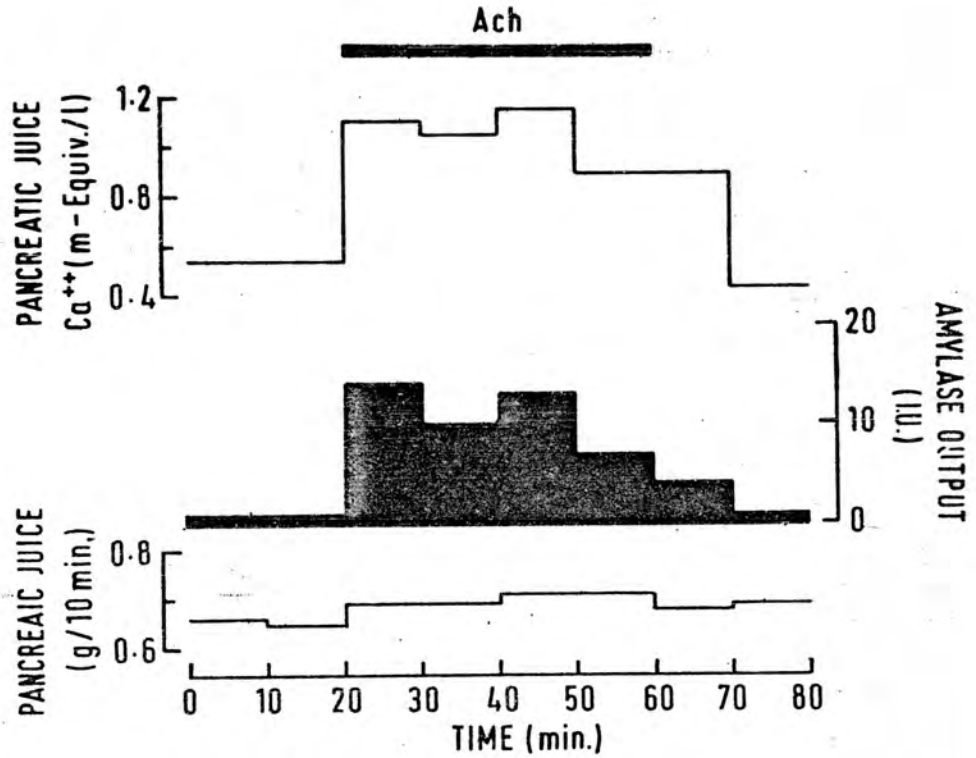


FIG. 7

Parallel output of calcium and amylase during acetylcholine (ACh) infusion. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min). ACh was infused at a rate of 20 ng/min for the duration of the horizontal bar.

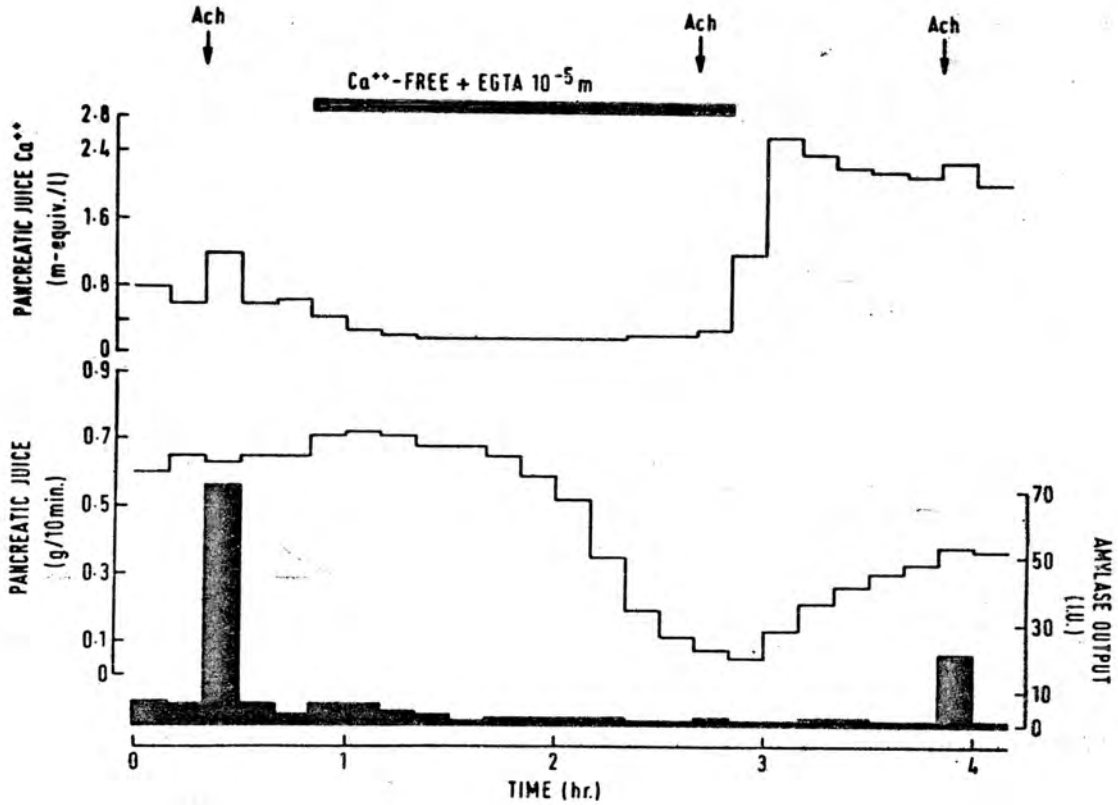


FIG. 8

The effects of prolonged perfusion with a calcium-free solution on electrolyte and amylase secretion from a perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin (30 μg/min). A Ca-free solution containing EGTA (10^{-5}M) was perfused through the gland for the duration of the horizontal bar. The arrows marked ACh indicate single injection of acetylcholine (200 ng).

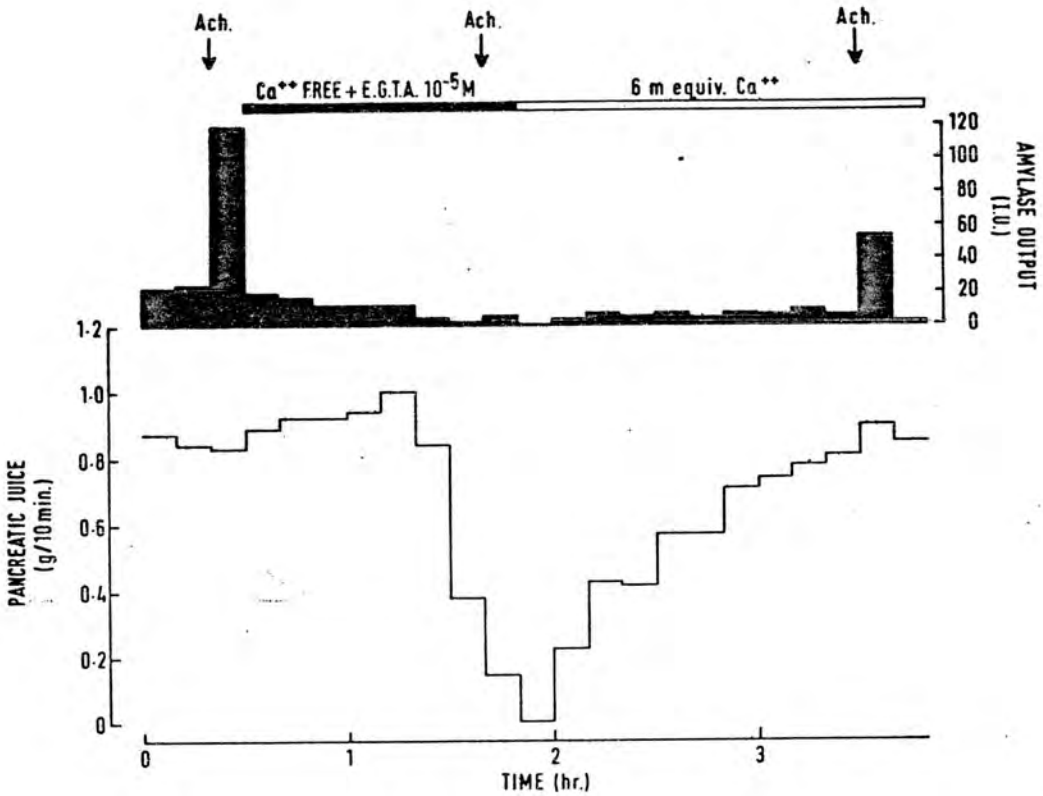


FIG. 9

The effect of Ca-rich perfusate on the recovery of electrolyte secretion following perfusion of the gland with a Ca-free solution containing EGTA (10^{-5}M). Electrolyte secretion was maximally stimulated throughout by infusing secretin at a supramaximal dose (30 µg/min). The calcium concentration of the perfusion fluid varied from normal (2.5 m Eq/l) for the periods indicated by the length of the horizontal bars. The arrows marked ACh indicate single injections of acetylcholine (1 µg).

the effect had become marked, perfusion with calcium-rich fluids (6-10 m-equiv Ca/l) was necessary for complete reversal (Figs. 8 and 9). By increasing the EGTA concentration of calcium-free perfusates, the time required for inhibition to become apparent was reduced (Fig.10). With a solution containing 10^{-3}M EGTA, the secretory rate in two experiments was reduced by a mean of 72% after only 20 mins. EGTA itself was not responsible for the inhibition for when added to normal (calcium containing perfusates), in concentrations up to 10^{-3}M, it had no inhibitory effects (two experiments) (Fig. 11).

Although reduced, basal enzyme secretion was always detectable during perfusion with calcium-free EGTA solutions, provided electrolyte secretion was maintained (Figs. 9 and 12). During either the initial period of calcium-free perfusion (when electrolyte secretion was unaffected)or the early phase of reduced secretory rate the response to acetylcholine was slightly diminished (Fig. 12). During the latter stages of reduced electrolyte secretion the response to acetylcholine was abolished (Figs. 8 and 9) and unlike electrolyte secretion it was not fully restored by perfusion with Ca-rich fluids (Fig. 9).

On returning to normal perfusate after prolonged calcium free perfusion the concentration of calcium in pancreatic juice was elevated when compared to the control period and often equalled the concentration in the perfusing fluid (Fig. 8).

Calcium-free solutions had no effect on the rate of perfusate flow through the gland. In this series of experiments similar results were obtained when CCK-PZ and pure secretin were substituted for acetylcholine and crude secretin.

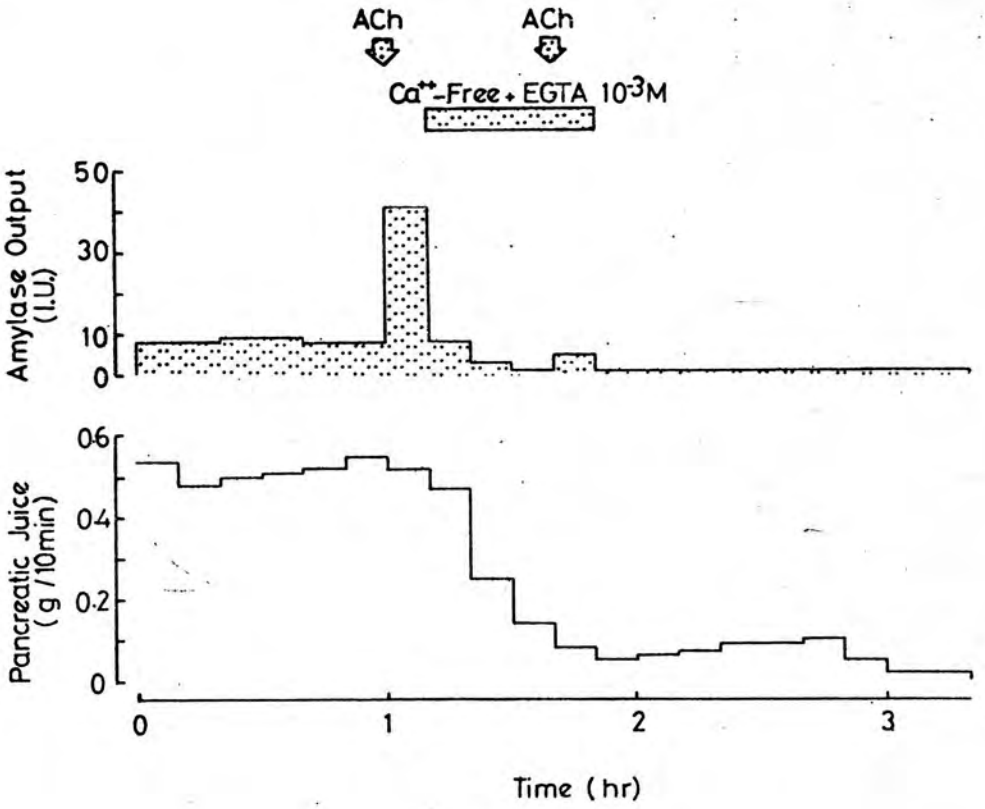


FIG. 10

The effect of a calcium-free perfusate on amylase and electrolyte secretion from a perfused cat pancreas. Electrolyte secretion was maximally stimulated throughout by infusing secretin at a supramaximal dose (30 µg/min). A calcium free solution containing EGTA (10^{-3}M) was perfused through the gland for the duration of the horizontal bar. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

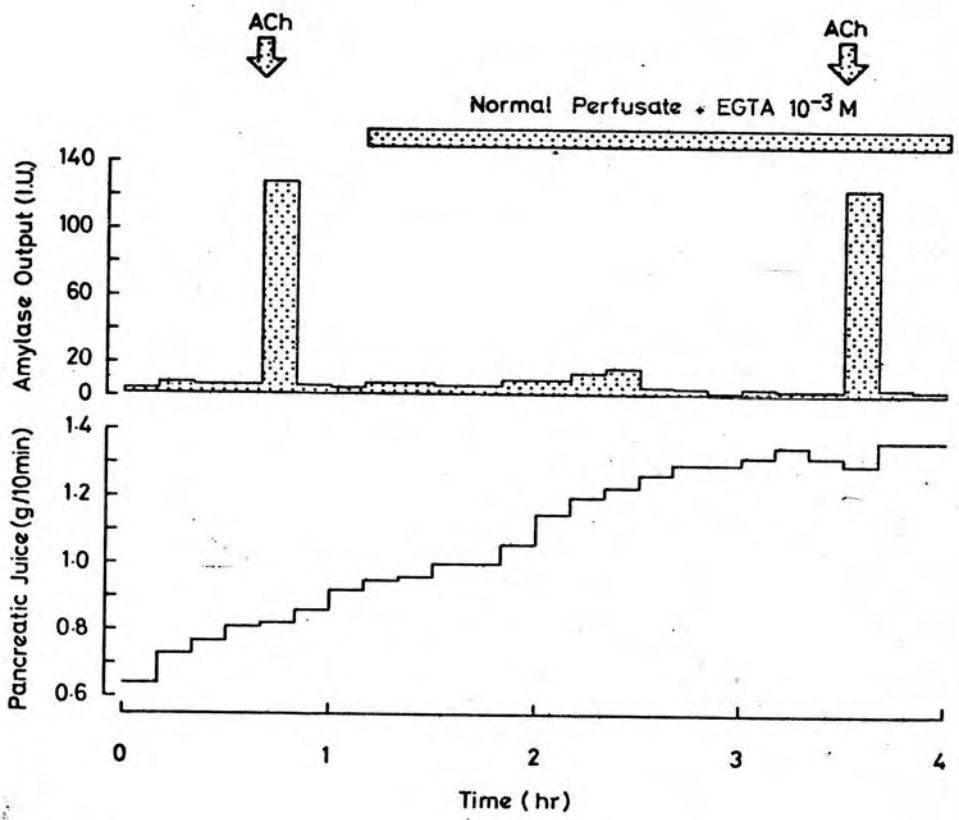


FIG. 11

The effect of adding EGTA (10^{-3}M) to normal perfusion fluid (Ca = 2.5 mEq/l) on electrolyte and amylase secretion from a perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing secretin at a supramaximal rate (30 µg/min). The arrows marked ACh indicate single injections of acetylcholine (1 ug)

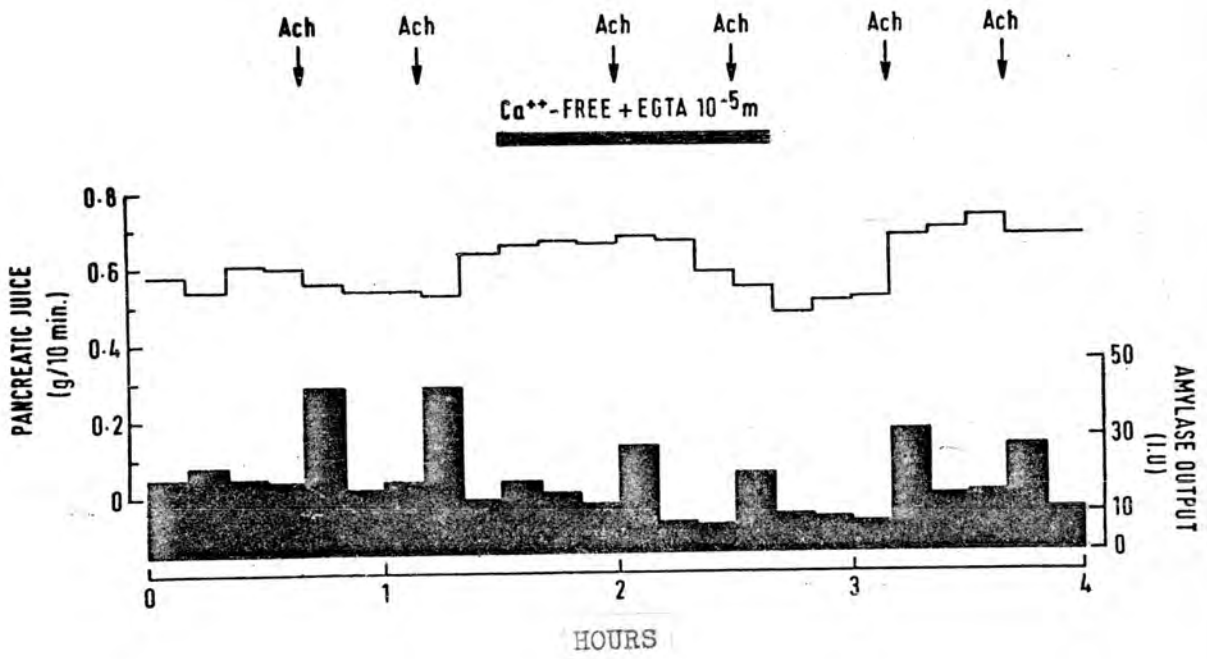


FIG. 12

The effect of calcium-free perfusate on electrolyte and amylase secretion from a perfused cat pancreas. Electrolyte secretion was maximally stimulated throughout by infusing secretin at a supramaximal dose (30 µg/min). A calcium-free solution containing EGTA 10^{-5}M was perfused through the gland for the duration of the horizontal bar. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

(2) The effects of calcium-rich perfusates on pancreatic secretion: Perfusion with fluids containing 10 m-equiv Ca/l. did not affect the rate of pancreatic secretion stimulated maximally or submaximally by secretin, but did cause the juice calcium concentration to rise and remain elevated throughout the 30 min. test period (Fig. 13). In three of seven such experiments a small increase (mean 120%) in basal amylase secretion was also observed on switching to a calcium rich buffer, but the effect was transient, being observed only in the first 10 min. period of perfusion.

In three further experiments, increasing the calcium concentration of the perfusate during minimal acetylcholine infusion (5 ng/min) caused an increase in amylase secretion (Fig. 14). This effect was not wholly due to the release of acetylcholine from nerve terminals within the gland, since in 3 experiments it was also observed during stimulation by CCK-PZ (2.5×10^{-2} Crick-Harper-Raper u./min) in the presence of atropine (10 mg/l).

(3) The effects of barium on pancreatic secretion: In two experiments barium (2.5 - 5.0 m-equiv/l) did not prevent the reduction in electrolyte and amylase secretion associated with prolonged calcium-free perfusion or aid the recovery of electrolyte secretion after calcium depletion.

C. Alterations in perfusate Mg

(1) The effect of magnesium-rich perfusate on pancreatic secretion: Perfusion with magnesium-rich fluids (10 m-equiv Mg/l) for up to 60

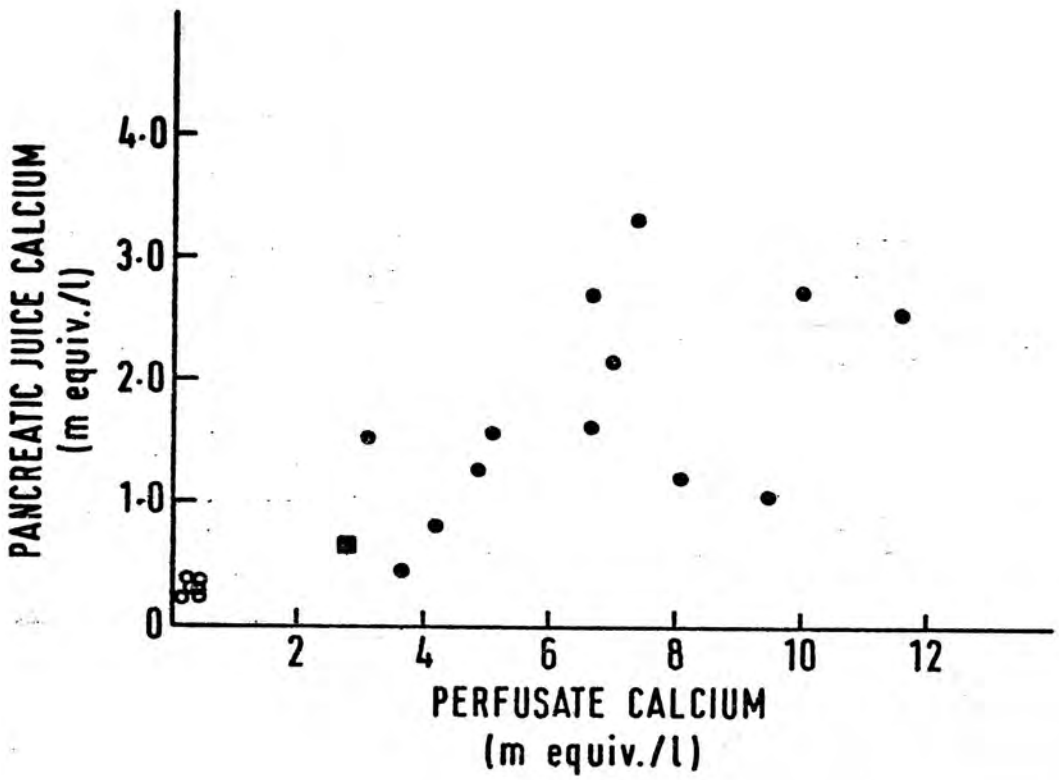


FIG. 13

The relationship between the concentration of calcium in perfusate and pancreatic juice. The closed circles represent single observations from a total of three experiments in which the concentration of calcium in the perfusion fluid was varied. The open circles represent single observations from six experiments in which the gland was perfused with a calcium-free solution containing EGTA. The filled square represents seventy eight observations from eleven experiments in which the gland was perfused with normal perfusion fluid.

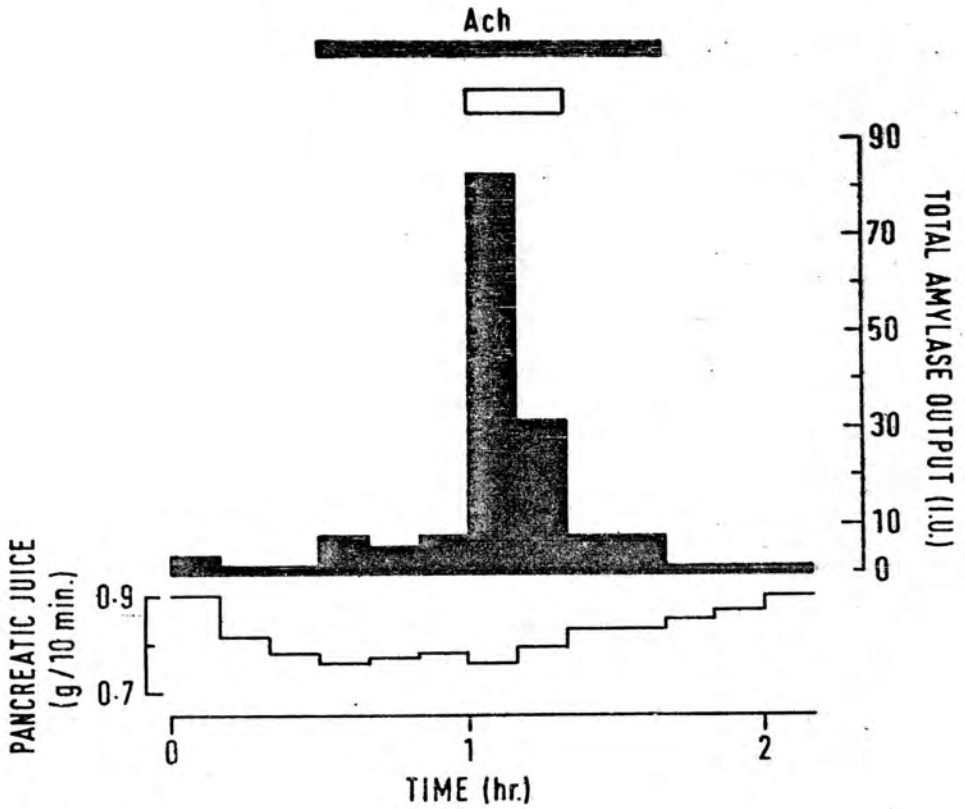


FIG. 14

The effect of increasing the perfusate calcium concentration during submaximal amylase secretion. Acetylcholine (5 ng/min) was infused into the gland for the period indicated by the filled bar. For the duration of the open bar the perfusate contained calcium at a concentration of 10 m equiv/l. Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supramaximal dose (30 µg/min)

min. was without marked effects on electrolyte or acetylcholine stimulated amylase secretion. Like calcium, excess magnesium caused a transient increase in basal enzyme secretion and potentiated minimal stimulation by acetylcholine, but the effects were never as great as those observed with calcium.

(2) The effect of magnesium-free perfusates on pancreatic secretion: Magnesium free solutions had no marked effects on electrolyte secretion or on acetylcholine stimulated enzyme secretion when perfused through the gland for up to 60 min. (two experiments).

D. Alterations in perfusate Na

(1) The effect of sodium deficient perfusates on pancreatic secretion.

Sodium deficiency is known to inhibit pancreatic electrolyte secretion (CASE et al. 1968). Its effect on enzyme secretion was tested in five experiments by perfusion with a solution containing either 0 or 50 mM-Na/l. After 50 mins. perfusion with 50 mM Na/l (Fig. 15), the response to acetylcholine was normal. However, because of the slow secretory rate the enzyme was not all eliminated from the duct system during the 10 min test period, most of it appearing in the period following return to normal perfusate. A return to normal perfusate alone produced only a minimal increase in enzyme secretion which presumably was a washing out of basal enzyme secretion that had accumulated in the ducts during the period of low electrolyte secretion.

When total replacement of sodium by sucrose was carried out the

experimental situation was complicated by several factors. Electrolyte secretion often ceased completely in these experiments and the large amount of sucrose required to maintain isosmolality caused an increase in the viscosity of the perfusate and a considerable fall in the rate of perfusate flow through the gland. In order to control these variables, the infusion of secretin was stopped during perfusion with the Na free solution and subsequently during a control period and the rate of perfusate flow through the gland during the control period was adjusted to equal that during the period of Na free perfusion. Of the four experiments in this series one showed no effect of Na depletion on acetylcholine stimulated amylase secretion, two demonstrated potentiations of +23% and +86% and one demonstrated an inhibition of -55%. The mean result would then be a potentiation of 57%. However, because of the wide variation, it is probably better to conclude that Na-free solutions had no consistent effect on acetylcholine stimulated enzyme secretion. This point requires further clarification in an experimental system where enzyme secretion can be studied in isolation.

E. Alterations in perfusate K

(1) The effect of potassium free solutions on pancreatic secretion: Potassium omission reduces pancreatic electrolyte secretion by about 60% (CASE et al. 1969b). However in three experiments, potassium free fluids did not affect acetylcholine-stimulated amylase secretion (Fig. 16). In these experiments an increase in basal enzyme and protein secretion was observed during potassium omission, an effect

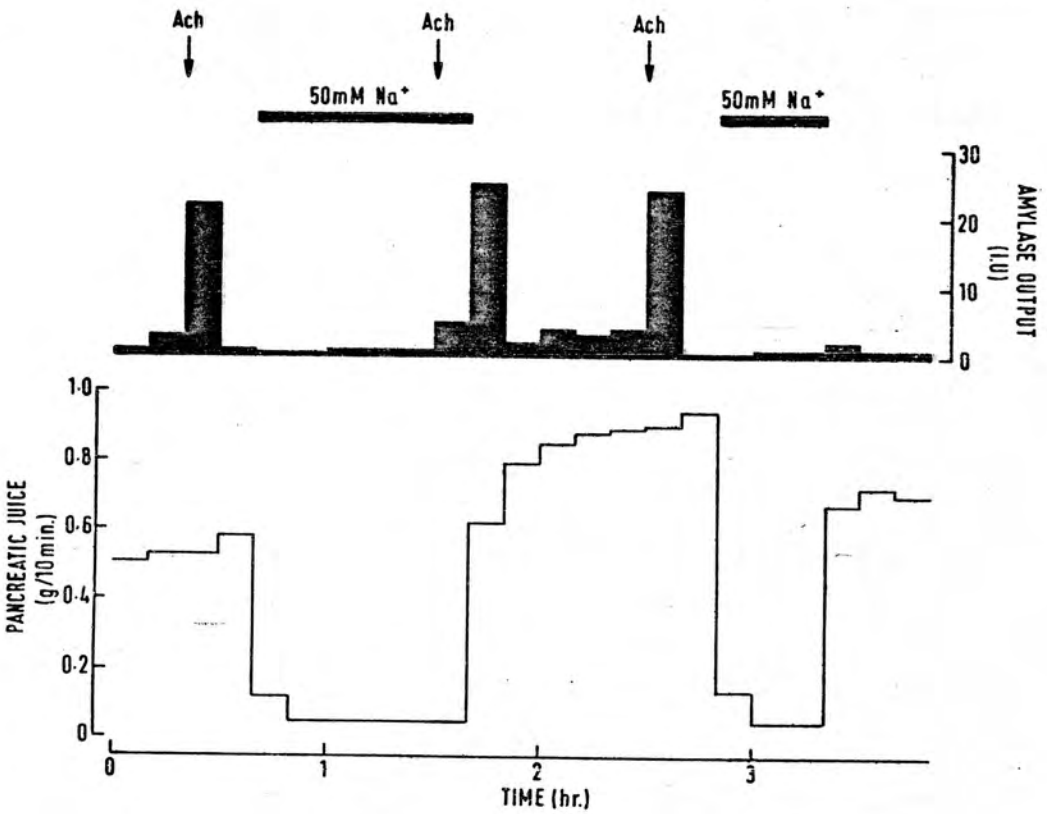


FIG. 15

The effects of sodium deficiency on electrolyte and amylase secretion from the perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min). For the duration of the horizontal bars the gland was perfused with a solution containing 50 mM Na/l isotonicity being maintained with sucrose. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

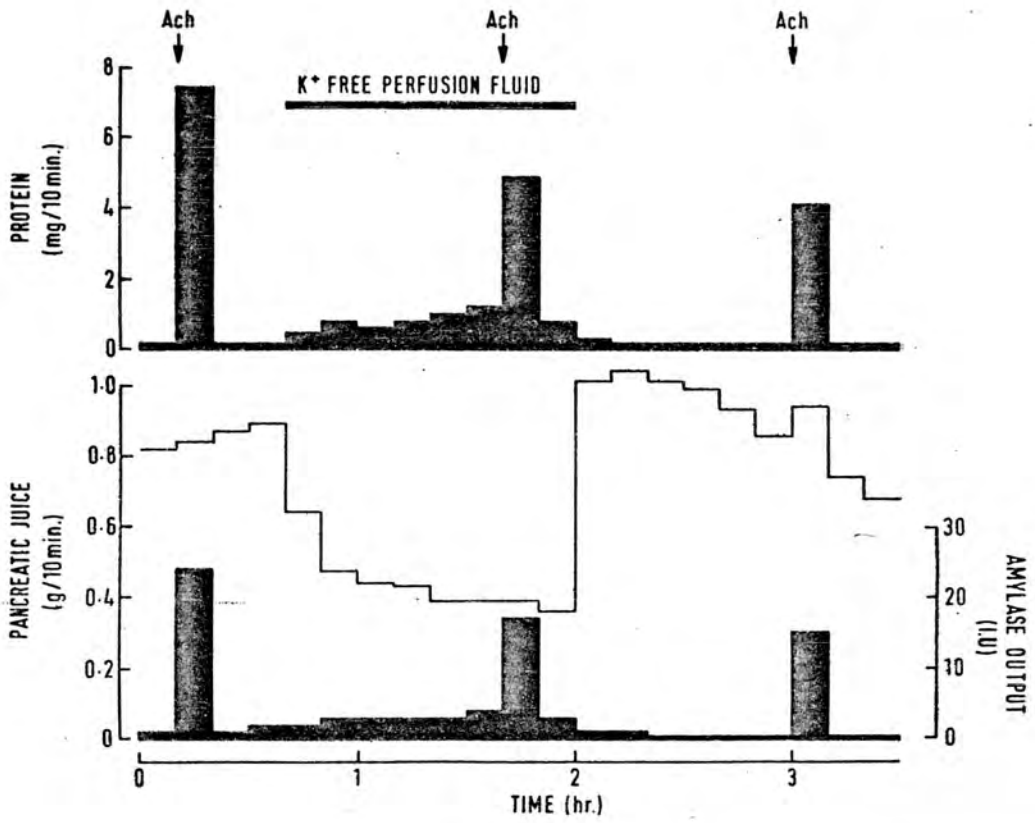


FIG. 16

The effects of potassium-free perfusion fluid on amylase, protein and electrolyte secretion from a perfused cat pancreas. Electrolyte secretion was stimulated throughout by infusing crude secretin at a supramaximal dose (30 μg/min). For the period denoted by the horizontal bar potassium was absent from the perfusion fluid. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

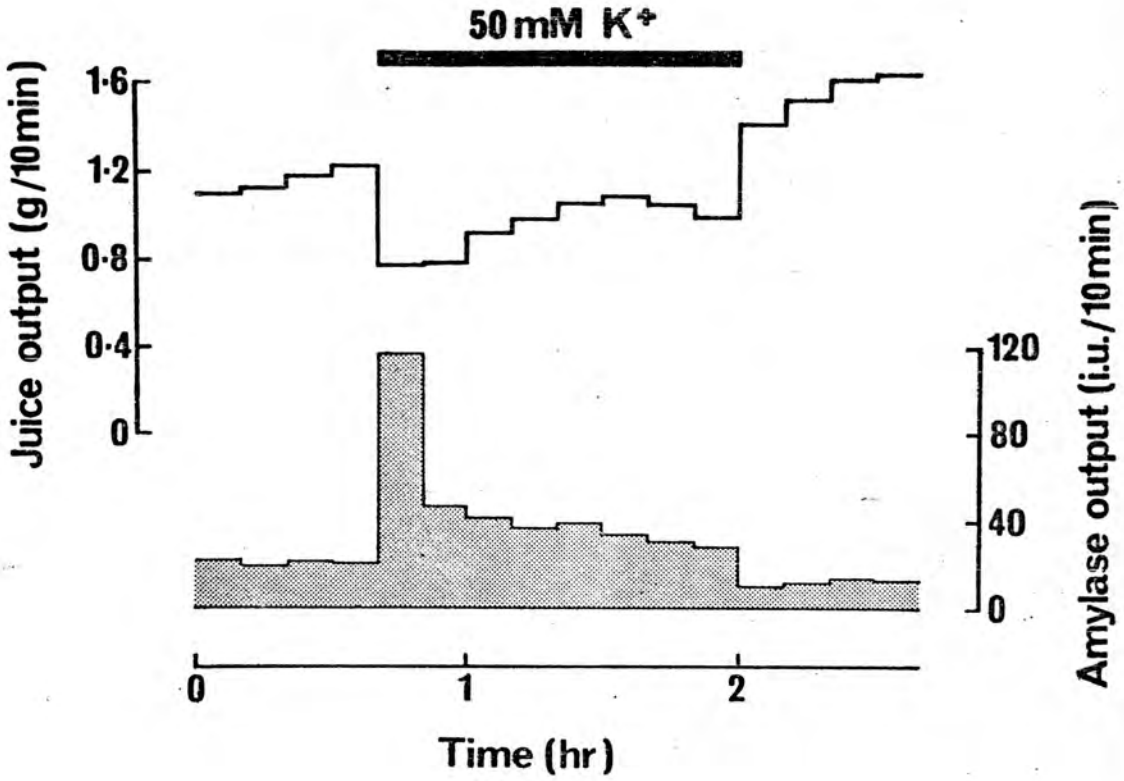


FIG. 17

The effect of replacing extracellular sodium with potassium on electrolyte and amylase secretion from an isolated perfused cat pancreas. Electrolyte secretion was maximally stimulated throughout by infusing secretin at a supramaximal dose (7.6 µg/min). For the duration of the horizontal bar the normal perfusate was replaced by one containing 50 mM potassium per litre.

which was blocked by atropine.

(2) The effect of potassium-rich perfusates on pancreatic secretion: In thirty six experiments, perfusion of the isolated cat pancreas with solutions containing potassium at concentrations of 30-120 mM for periods of 5-80 mins stimulated amylase secretion (Fig. 17). The amylase output was greatest during the first few minutes of exposure to excess potassium and remained above basal levels throughout the test period.

Potassium also caused a reduction in the rate of perfusate flow through the gland and increased the perfusion pressure presumably due to vasoconstriction (Fig. 18). Noradrenaline infusion produced similar effects (Fig. 19). In two experiments the α -blocking agent phenoxybenzamine (10 mg/l) lessened this potassium-induced reduction in perfusate flow by a mean of 54%.

Accompanying the enzyme secretion, potassium also caused a reduction in the rate of electrolyte secretion (Fig. 17), which remained depressed until a return to normal perfusion fluid was made after which the rate was often elevated compared with the control period.

Since an increase in perfusate potassium concentration was always accompanied by an equivalent decrease in sodium concentration, the effect of replacing sodium chloride with isosmotic amounts of sucrose was investigated. This procedure is known to decrease the volume of pancreatic secretion (CASE et al.1968). The decrease in secretory rate was linearly related to the decrease in sodium concentrations, and was not significantly different when potassium chloride was used instead of sucrose (Fig. 20). However replacing sodium chloride with

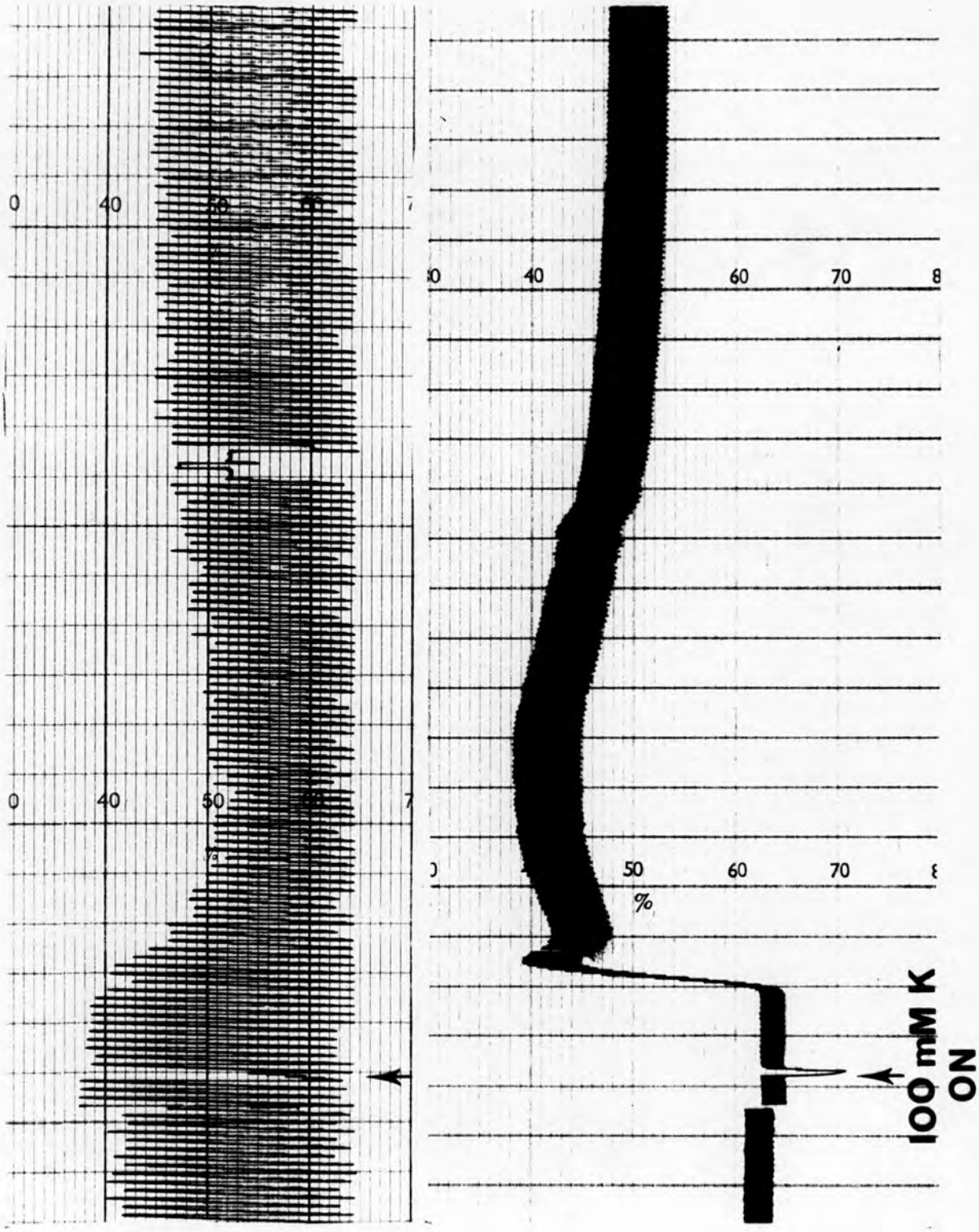


FIG. 18 (PART 1)

The effects of increasing the concentration of potassium in the perfusion fluid to 100 mM on perfusion pressure and flow rate.

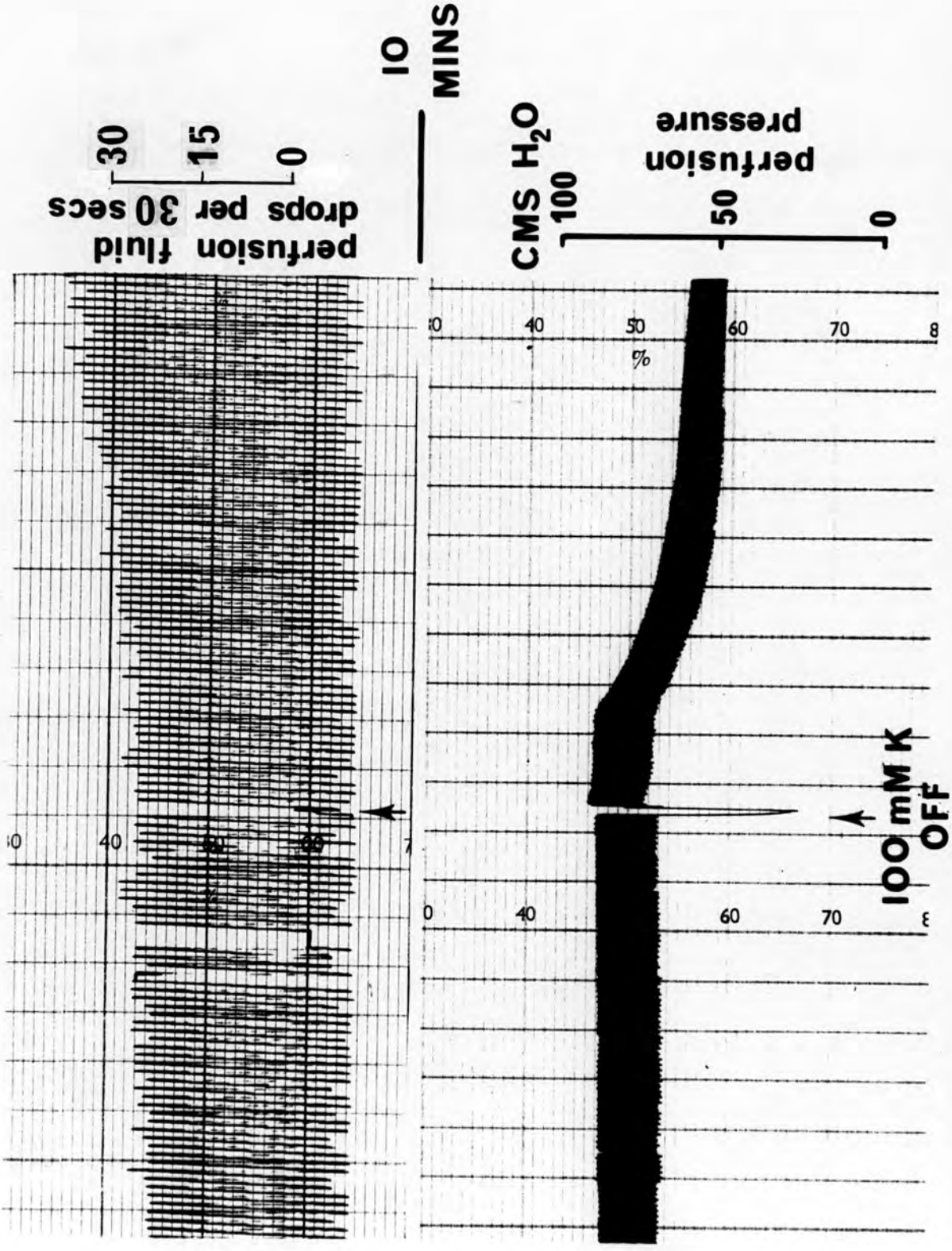


FIG. 18 (PART 2)

Continuation of FIG. 18

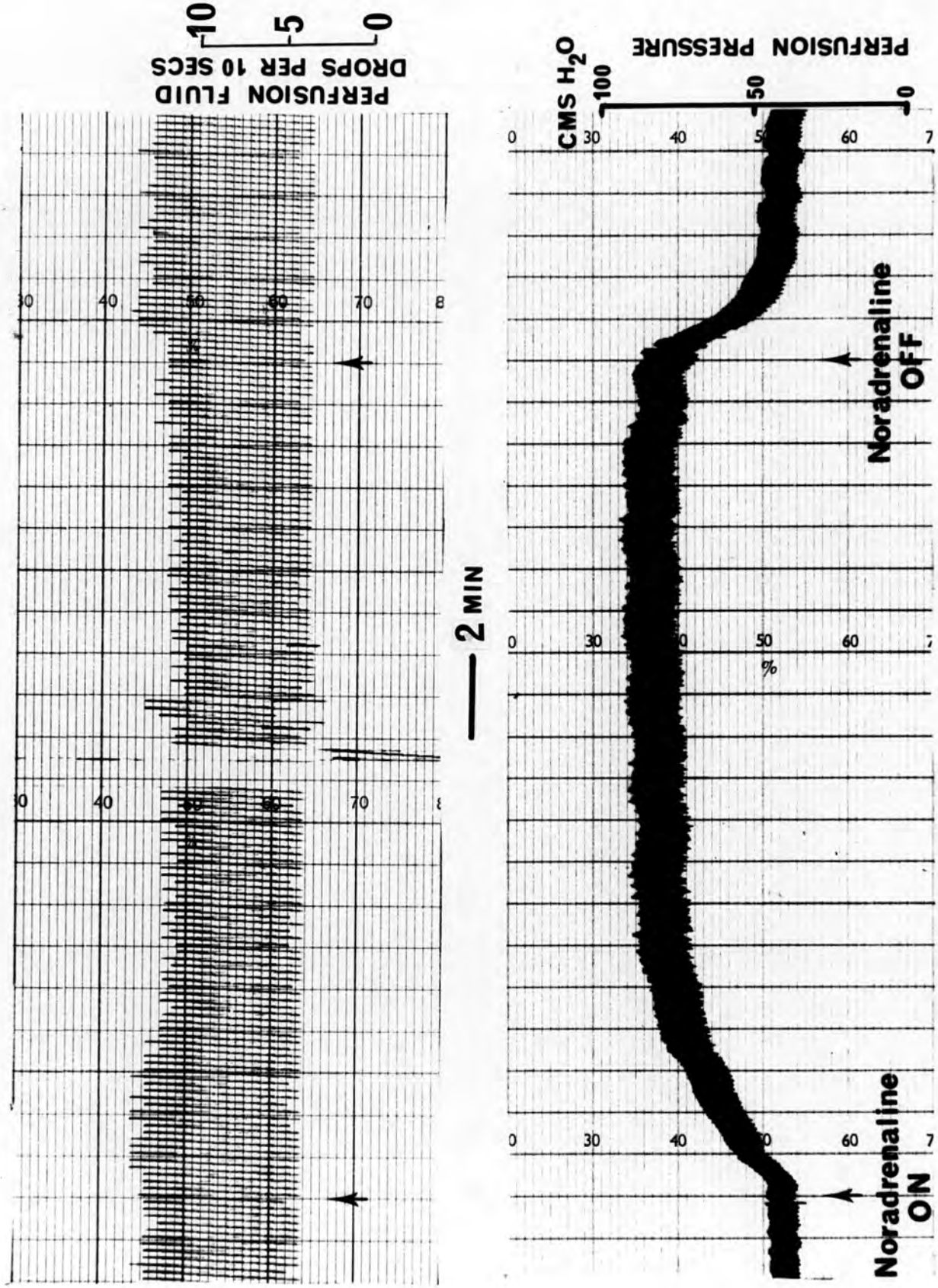


FIG. 19

The effects of noradrenaline infusion (1 µg/min) on perfusion pressure and flow rate.

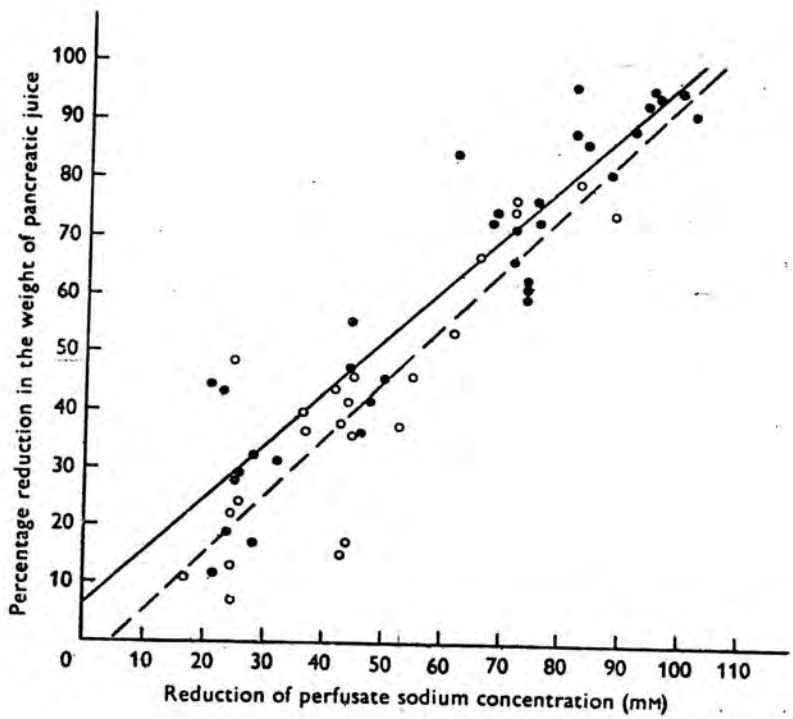


FIG. 20

The effect of reducing the perfusate sodium concentration on the volume of pancreatic juice secreted by the isolated cat pancreas. The open circles represent twenty-three observations in fifteen experiments in which the perfusate sodium chloride was replaced by potassium chloride; the interrupted line is the calculated regression line of these observations. The filled circles represent thirty-five observations in ten experiments from an earlier study (CASE et al.1968) where sodium chloride was replaced by an osmotically equivalent amount of sucrose: the continuous line is the calculated regression line of these observations. Each point was obtained by expressing the response at equilibrium to the test solution as a percentage of that to normal perfusate. The regression lines which are both statistically significant ($P < 0.05$) do not differ from each other ($P > 0.05$).

sucrose did not stimulate enzyme secretion nor cause vasoconstriction.

(3)The stimulation of enzyme secretion in relation to potassium concentration: The enzyme stimulating effect of potassium was examined in detail in five experiments. In each experiment four solutions each containing a different concentration of potassium were perfused in random order, through the gland for the first 5 min. of a 10 min. collection period. The amylase output during each test period was expressed as a percentage of the response to a standard dose of acetylcholine (5 µg) given at the end of the experiment. In each experiment the first response was ignored because it was always greater than subsequent stimulations as shown in control experiments where the same potassium concentration was tested repeatedly. The observations from all experiments are combined in Fig. 21 which also illustrates observations from three further experiments in which a total of five different potassium concentrations were tested in a similar way. No enzyme secretion occurred with potassium concentrations below 30 mM. The maximal output of enzyme was observed at potassium concentrations of 80-90 mM. Above this concentration output tended to decline and at 120 mM (the highest concentration tested) the response was not significantly different from that at 50-60 mM.

(4) The effect of atropine on amylase secretion stimulated by potassium: Atropine was used in order to test whether the stimulation of amylase secretion by potassium-rich solutions was due to a direct action of potassium on the acinar cell or to the stimulation of cholinergic nerves in the gland. In eleven experiments the addition of atropine sulphate (7.0×10^{-8} - 1.4×10^{-5}M) abolished the enzyme

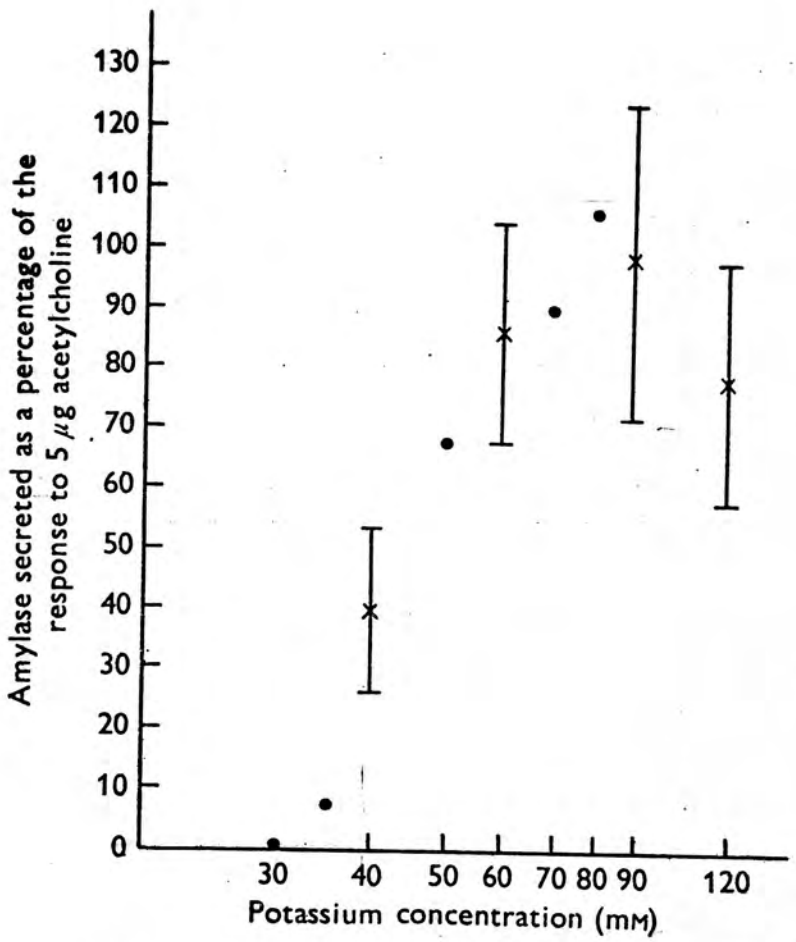


FIG. 21

The effect of potassium-rich perfusates on amylase secretion from the isolated cat pancreas. The total amount of amylase secreted in response to a 5 min perfusion with a given potassium concentration is expressed as a percentage of the response to 5 μg acetylcholine. The order in which the various concentrations of potassium were tested in different experiments was random. X = mean (± S.E. of mean) of five observations from five experiments • = mean of two observations from a total of three experiments.

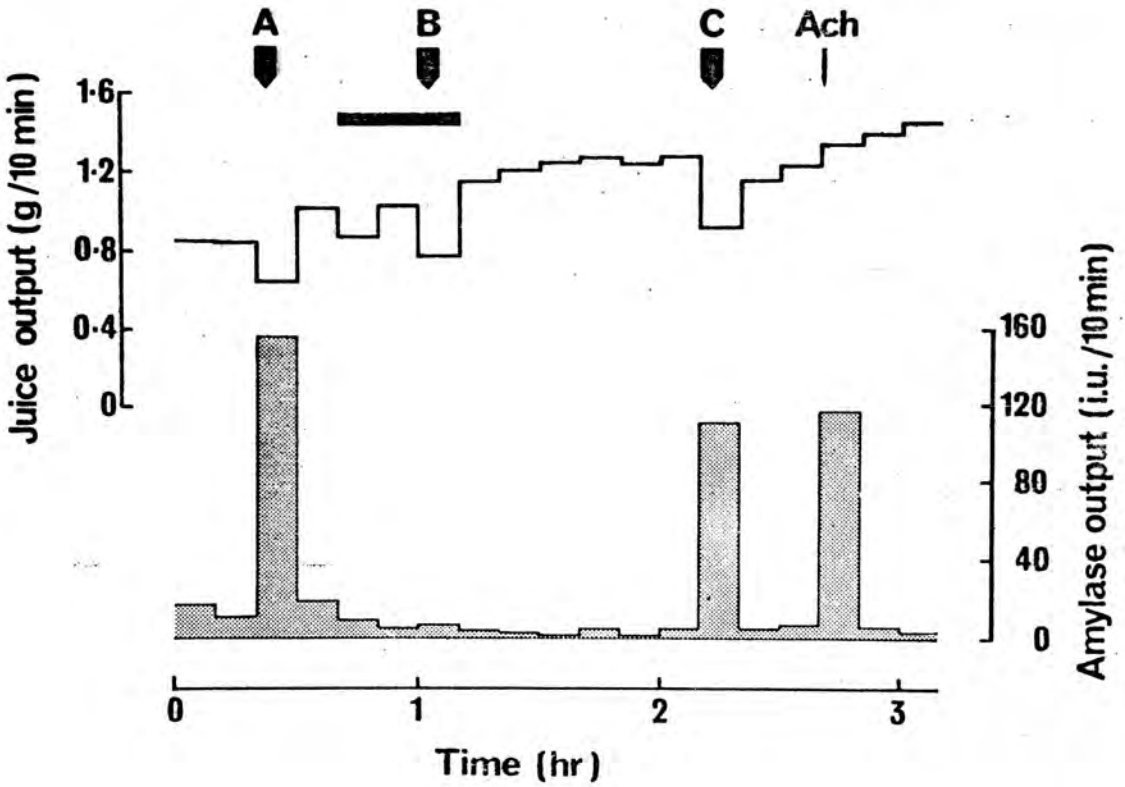
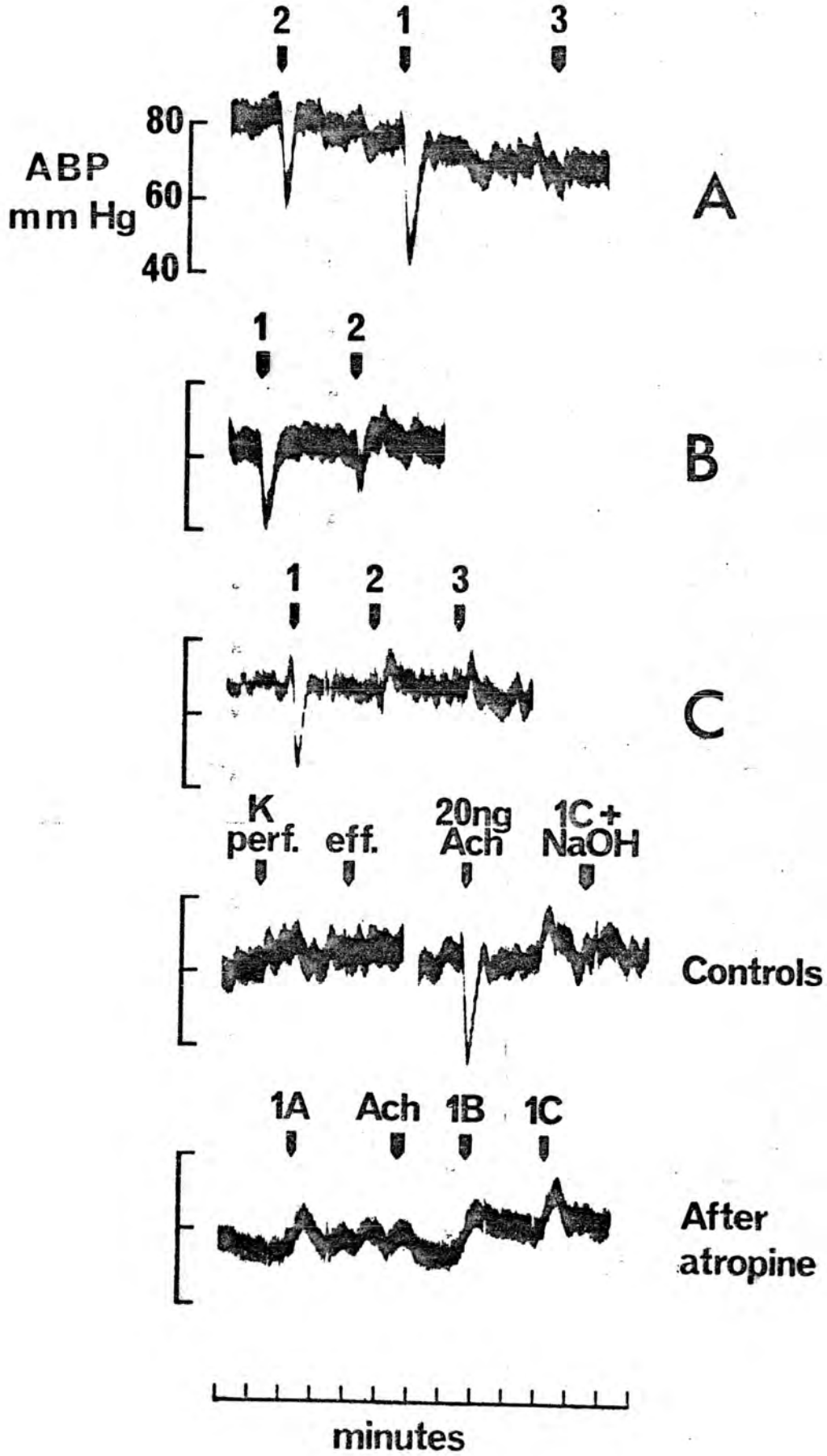


FIG. 22

The effect of atropine on electrolyte and amylase secretion from a perfused cat pancreas in response to excess potassium. Electrolyte secretion was stimulated maximally throughout by infusing secretin at a supramaximal dose (7.6 µg/min). The perfusion fluids contained atropine sulphate (1.4×10^{-7} M) for the length of the horizontal bar. The broad arrows lettered A, B and C indicate 5 min periods during which the perfusate contained 50 mM potassium. The arrow marked Ach indicates a single injection of 5 µg acetylcholine.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Controls
1A
Ach
1B
1C
After
atropine
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FIG. 23

FIG. 23

The detection of acetylcholine (Ach) in the effluent from a perfused cat pancreas. Ach was assayed by its depressor action on the arterial blood pressure of the same animal in which the perfusion experiment was performed. The effluents tested were those collected in the experiment illustrated in Fig. 22. During each 5 min perfusion with 50 mM potassium (lettered A, B and C; see Fig. 22) the total volume of effluent was collected and labelled sample I. The effluent collected in the two succeeding 5 min periods (during perfusion with normal perfusate) were labelled sample 2 and 3 respectively. The effect of 1.0 ml aliquots of all these samples is shown in the upper three blood pressure traces. A series of controls shows the effect of injecting potassium rich perfusate (K perf), effluent of normal composition (eff), a 20 ng dose of Ach (20 ng Ach) and a sample of potassium-rich effluent after treatment with sodium hydroxide (1C + NaOH). The lowest trace illustrates the effect of the potassium-rich effluents and Ach (20 ng) after the cat had been given atropine sulphate (0.8 mg/kg). ABP: arterial blood pressure.

secretion in response to potassium, though the reduction in pancreatic juice volume persisted. Fig. 22 illustrates an experiment in which the isolated pancreas was perfused with a potassium rich solution (50 mM) which stimulated a copious secretion of amylase. Atropine (1.4×10^{-7}M) abolished the effect on enzyme secretion. One hour after the cessation of atropine infusion potassium (50 mM) again stimulated the secretion of amylase. Atropine itself had no effect on the basal secretion of amylase. When the more specific antimuscarinic drug hyoscine (2.3×10^{-7}M) was substituted for atropine similar results were obtained.

(5) <u>The presence of a vasodepressor material (acetylcholine) in the effluent from the gland</u>: The inhibition of enzyme secretion by atropine suggested that potassium acts by releasing acetylcholine from nerve terminals. Further evidence that acetylcholine is the mediator of the response was obtained from analysis of the effluent from the gland. In seven experiments samples of effluent were collected before, during and after perfusing the gland with a potassium-rich solution. Aliquots of each sample (0.5 or 1.0 ml) were injected into the cat's jugular or saphenous vein and changes in blood pressure monitored. All experiments gave similar results to those illustrated in Fig. 23, which is from the same experiment as Fig. 22. The injection of effluent collected during 5 min perfusion with 50 mM potassium caused a fall in blood pressure, approximately equal to that obtained by injecting 20 ng acetylcholine. Vasodepressor material was also present in effluent collected during the 5 min period immediately after perfusion with 50 mM potassium but not in the subsequent collection (Fig. 23 A). Similar observations were made

after atropine (1.4×10^{-7}M) had been added to the perfusion fluid (Fig. 23B) although atropine abolished the amylase secretion in response to potassium (Fig. 22). During the third period of stimulation with 50 mM potassium, after atropine had been removed from the perfusion fluid, the vasodepressor material was again detected in the effluent from the gland (Fig. 23C).

Effluent of normal composition had no effect on blood pressure. Potassium-rich perfusion fluid (i.e. before entering the gland) caused a slight rise in blood pressure. The depressor effect of active effluents was abolished either by making them alkaline with sodium hydroxide, or by atropinizing the cat (0.8 mg/kg). After these procedures the effluents had a pressor effect slightly greater than that obtained with potassium-rich perfusion fluid.

These observations suggest that acetylcholine is the depressor substance in the effluent, and is presumably released from nerve terminals within the gland by the high potassium concentrations. The pressor responses observed after blocking the depressor activity suggests that potassium may also release small amounts of catecholamines from sympathetic nerve terminals within the gland.

These results indicate that the secretory process of the acinar cell is unaffected by high extracellular potassium concentrations. In seeking support for this view, the effect of potassium rich perfusion fluid on CCK-P2 stimulated enzyme secretion was tested in three experiments. The perfusion fluid contained atropine sulphate (10 mg/l) to block the action of released acetylcholine. The response to CCK-P2 was virtually unaffected by high extracellular potassium concentrations (Fig. 24).

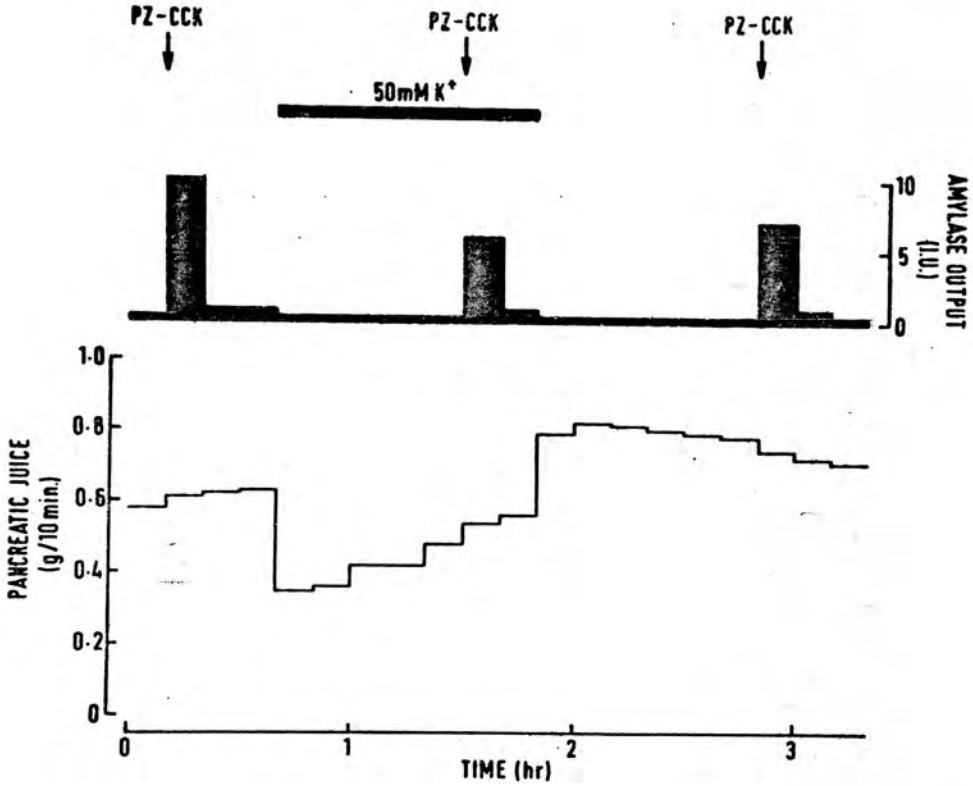

FIG. 24

The effect of excess potassium on amylase secretion from the perfused cat pancreas. For the duration of the horizontal bar the gland was perfused with a solution containing 50 mMK. The arrows indicate single injections of pure CCK-PZ (1.0 C.H.R. Unit). To prevent the action of acetylcholine released by potassium from nerve terminals within the gland all perfusion fluids contained atropine (10 mg/l.). Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supramaximal dose (30 µg/min).

	40mM			90mM		
	Rb	Cs	K	R6	Cs	K
Mean	64.75	113.3	39	71.75	174.3	97.4
± S.E. of mean	± 15.48	± 17.11	± 13.5	± 19.72	± 38.3	± 26.26

Amylase secreted is expressed as a % of the response to 5 µg acetylcholine.

Table I Effects of Rubidium, Caesium or Potassium at concentrations of 40 and 90mM on Secretion of Amylase from the perfused pancreas.

(6) The stimulatory effect of other alkali metal ions on amylase secretion: Several experiments were performed to ascertain whether amylase secretion could be evoked by alkali metal ions other than potassium.

In three experiments lithium at concentrations of 30-120 mM did not stimulate the secretion of enzymes from the pancreas. Rubidium and caesium however had effects similar to potassium. In four experiments the effects of rubidium and caesium were compared at two concentrations 40 and 90 mM. (TABLE 1). The design of the experiments was similar to those used in testing different concentrations of potassium, the response to each test fluid (given in random order) being compared with the response to 5 µg acetylcholine (given last). At both concentrations the mean amylase output in response to caesium was greater than that in response to rubidium and potassium although only the difference between potassium and caesium at 40 mM was statistically significant ($P < 0.02$). The response to rubidium and potassium did not differ significantly at either concentration ($P > 0.1$).

Lithium, rubidium and caesium all reduced the volume of pancreatic secretion but only rubidium and caesium significantly reduced the rate of perfusate flow through the gland. The reduction in perfusate flow observed with caesium was usually less than that observed with either potassium or rubidium.

F. The effects of tetracaine (amethocaine), colchicine and nystatin on pancreatic secretion.

(1) Tetracaine (amethocaine): In two experiments perfusing the isolated pancreas with solutions containing the local anaesthetic tetracaine ($2.0 - 5.0 \times 10^{-4}$M) reduced the secretion of amylase in response to single doses of acetylcholine but had no effect on the volume of pancreatic secretion (Fig. 25). In three further experiments concentrations of tetracaine greater than 10^{-3}M caused a reduction in the volume of pancreatic secretion (Fig. 26).

(2) Colchicine : In two experiments the antimitotic drug colchicine (2mM) was perfused through the isolated pancreas for up to 3 hrs. In one of these experiments colchicine caused an inhibition of both basal and acetylcholine stimulated amylase secretion which was only partially reversible (Fig. 27). In both experiments colchicine caused a progressive increase in the rate of electrolyte secretion, stimulated by infusion of secretin at a supramaximal dose. This effect is marked in the experiment illustrated in fig. 28 where the electrolyte secretory rate doubled (0.68 - 1.36 g/10 min) during a 160 min perfusion with 2mM colchicine.

(3) Nystatin: In one experiment Nystatin (1.5×10^{-4}M) caused a reduction in the rate of electrolyte secretion and inhibited acetylcholine stimulated amylase secretion by approx. 50% (Fig. 29).

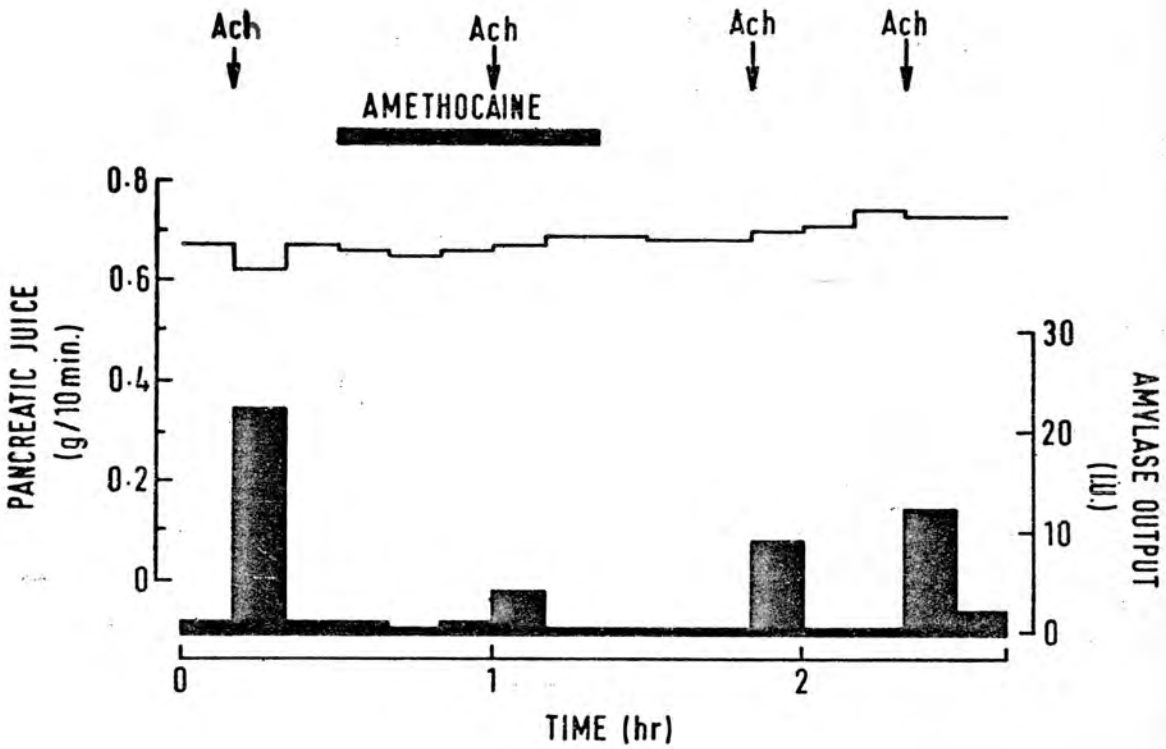

FIG. 25

Effects of a low dose of the local anaesthetic tetracaine (amethocaine) on amylase and electrolyte secretion by a perfused cat pancreas. For the period denoted by the horizontal bar the perfusion fluid contained tetracaine (2.0×10^{-4}M). The arrows marked Ach indicate single injections of 200 ng acetylcholine. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 µg/min).

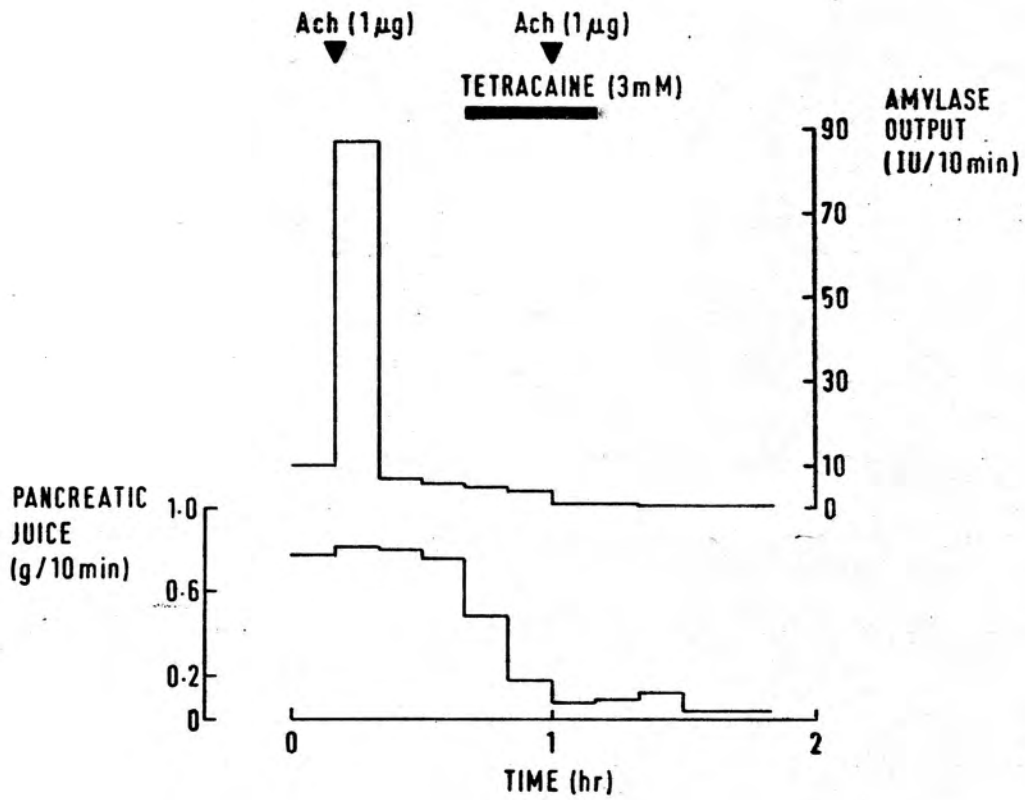

FIG. 26

Effects of a large dose of the local anaesthetic tetracaine on electrolyte and amylase secretion from a perfused cat pancreas. For the period denoted by the horizontal bar the perfusing solution contained tetracaine (3×10^{-3}M). The arrows marked Ach indicate single injections of 1µg acetylcholine. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 µg/min).

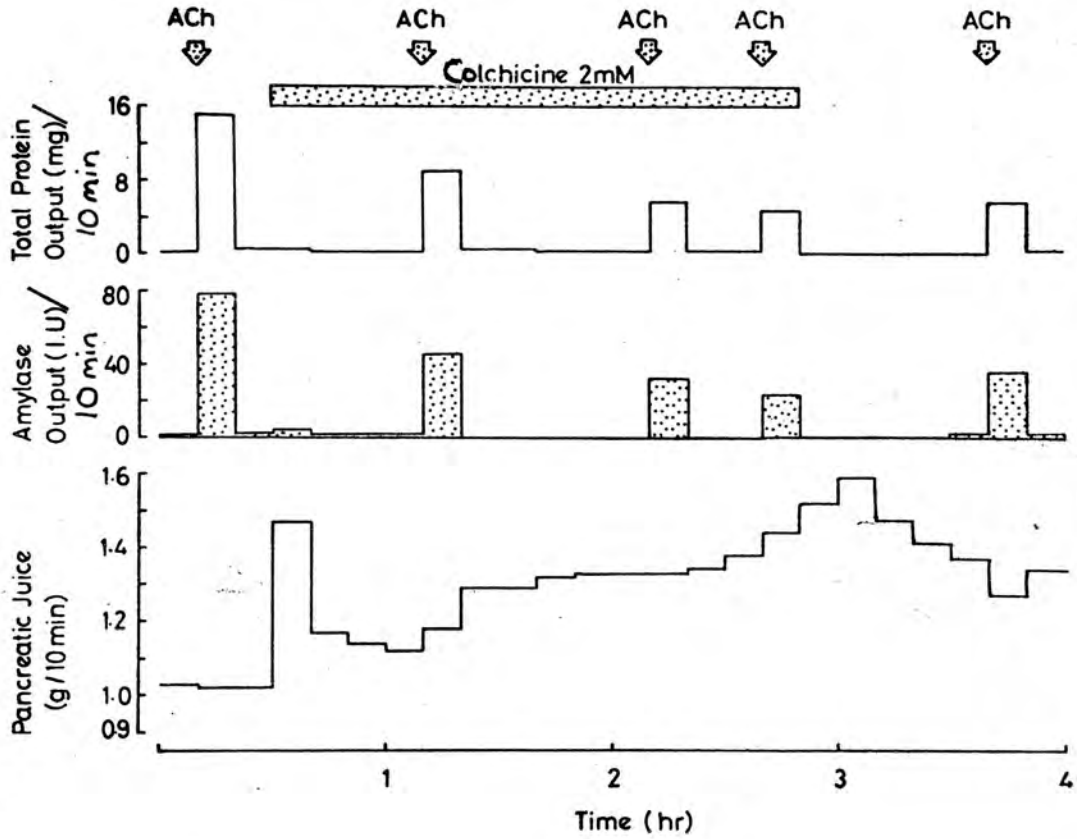

FIG. 27

The effects of colchicine on electrolyte and amylase secretion from a perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing secretin at a supramaximal dose (30 μg/min). For the duration of the horizontal bar the perfusion fluid contained colchicine (2 mM). The arrows marked Ach indicate single injections of acetylcholine (200 ng).

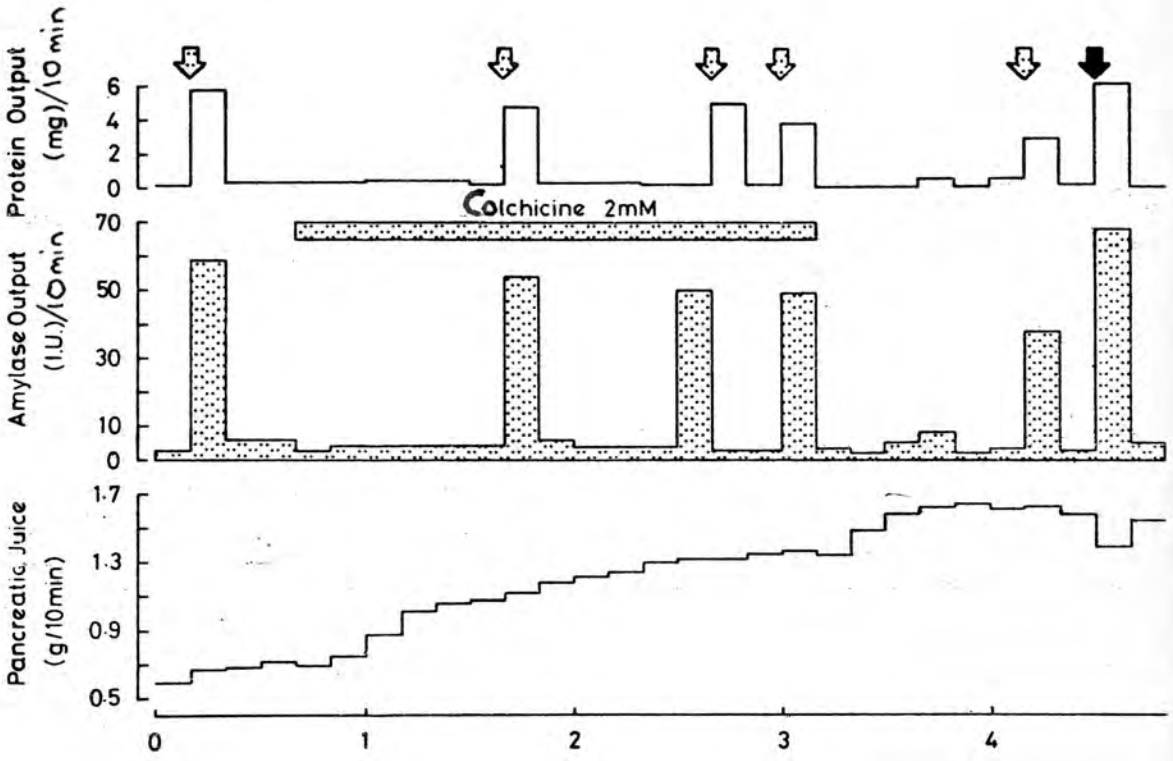

FIG. 28

The effects of colchicine on electrolyte and amylase secretion from the perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing secretin at a supramaximal dose (30 µg/min). For the duration of the horizontal bar the perfusion fluid contained colchicine (2 mM). The arrows indicate single injections of acetylcholine (200 ng) while the final thick arrow indicates a large dose (500 ng) of the same stimulant.

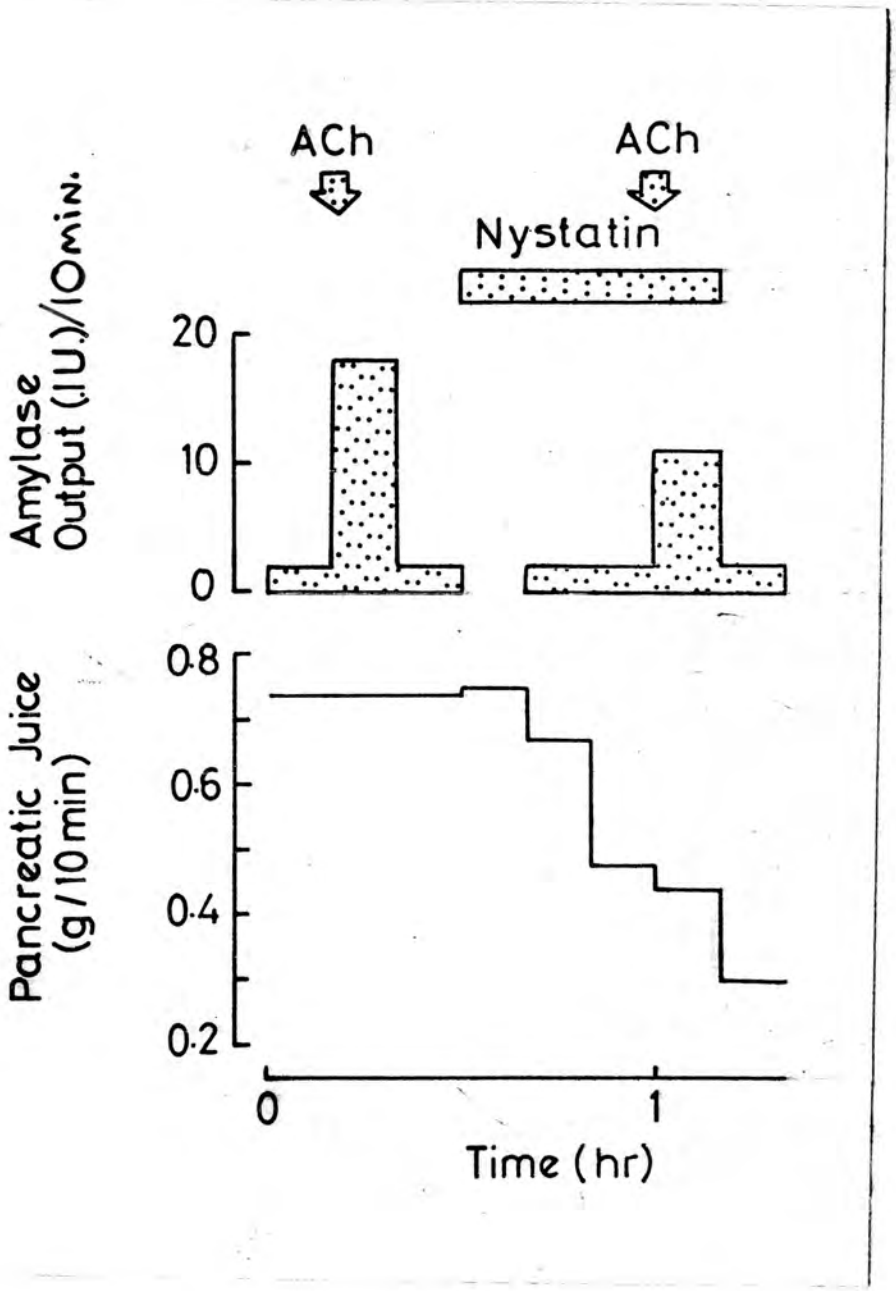

FIG. 29

The effects of nystatin on amylase and electrolyte secretion from a perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by the infusion of secretin at a supramaximal dose (30 µg/min). For the duration of the horizontal bar the perfusion fluid contained nystatin (1.5×10^{-4}M). The arrows marked ACh indicate single injections of acetylcholine (200 ng).

DISCUSSION

1. Enzyme secretion by the perfused cat pancreas

Previously the perfused preparation of the cat pancreas has been used to study electrolyte secretion. In their paper describing this technique CASE et al. (1968) demonstrated that secretin-stimulated electrolyte secretion collected from the *in vitro* gland was, with the exception of a falling bicarbonate concentration during the course of an experiment, similar in both rate and composition to that obtained from an intact anaesthetised cat. This was in spite of the fact that the *in vitro* gland rapidly becomes oedematous. The present studies show that amylase secretion from the isolated pancreas is also similar to that previously described for the gland *in vivo* (CASE et al. 1969a). Amylase activity is always detectable in pancreatic juice collected from the perfused gland and this small continuous basal enzyme secretion which is independent of nervous or hormonal control may be supplemented by the response to acetylcholine or CCK-PZ. In the absence of secretin-induced electrolyte secretion, the basally secreted enzyme accumulates in the duct system accounting for the 'wash out' phenomenon, that is a high concentration of enzymes in the first few drops of secretion obtained from a resting gland.

In common with the intact gland (CASE et al. 1969a) the response to a single dose of enzyme stimulant was an evanescent process. A rapid pulse of acetylcholine administered to a gland supramaximally stimulated by secretin elicited a response which reached a peak after 3 mins and was essentially completed after 5 mins. As the perfusion fluids did not contain amino acids the ability of the isolated gland to synthesize enzyme proteins must be limited by the size of the intra-

cellular pool of aminoacids. This may account for the decreasing enzyme output in response to repeated large doses of acetylcholine and CCK-P%. However the consistent response to repeated small doses validates the use of this preparation in studying pancreatic enzyme secretion. For the purposes of this study amylase output has been used as an index of total enzyme secretion since AGREN & LAGERLOF (1936) and BARRINGTON (1941) have shown that the three principle enzymes of pancreatic juice(protease, lipase and amylase) are secreted in parallel.

2. The concentration of calcium in pancreatic juice.

In the conscious dog the concentration of calcium in pancreatic juice varies inversely with the rate of secretion (Zimmerman, Dreiling, Rosenberg & Janowitz, 1967; Zimmerman, Moore, Dreiling & Janowitz, 1971). The calcium concentration is highest under conditions of basal secretion, but falls to values considerably lower than the plasma concentration when the gland is maximally stimulated by secretin.

This observation, together with the fact that the calcium concentration of the juice increased when the gland was stimulated to secrete enzyme has led these authors to conclude that calcium is not secreted with the electrolyte but rather with the enzyme component of pancreatic juice. The reduction in calcium concentration during secretin stimulation being due to the dilution of basally secreted enzyme.

The findings in the isolated cat pancreas in part confirm these earlier results. Under conditions of maximal secretin stimulation the calcium concentration in pancreatic juice (0.63 $\pm$ 0.04 m-equiv/l.) was considerably lower than that of the perfusing fluid (2.82 $\pm$ 0.09

m-equiv/l.). When the rate of pancreatic secretion was varied over a wide range it was impossible to detect any variation in the calcium concentration of the juice. However, in one experiment when the gland was stimulated to secrete at very slow rates below 0.15g/10 min an increase in the calcium concentration of the juice was detected and this correlated with an increase in the concentration of basally secreted amylase. When the gland was stimulated to secrete amylase by single injections or continuous infusion of acetylcholine or CCK-PZ both the concentration and output of calcium in the juice increased in proportion to the total amount of amylase secreted.

A close parallelism in the concentration of calcium and exportable protein in gastric (MOORE & MAKHLOUF, 1967) and salivary (DREISBACH, 1967; WALLACH & SCHRAMM, 1971) secretions supports the conclusion of these authors that calcium is bound to the digestive enzymes and may have its origins in the zymogen granules. Calcium is known to form an internal chelate in the salivary amylase molecule (STEIN, HSIU & RISCHER, 1964 a,b) and this may be so in the pancreas as is known to occur with zinc (PEKAS, 1971) and inorganic sulphate (BERG & YOUNG, 1971).

However not all calcium enters pancreatic juice bound to enzyme proteins. Perfusing the gland with a calcium-rich fluid caused the calcium concentration of the juice to rise without a parallel increase in enzyme concentration. Similar effects have been reported to occur during hypercalcaemic states in man (GOEBELL, STEFFAN, BALTZER & BODE, 1973). Also extrapolation of the regression line correlating juice calcium and amylase outputs (Fig. 6) indicates that at zero amylase output pancreatic juice still contains calcium. This second calcium component must arise either by way of the electrolyte secretory mechanism or by diffusion through the duct system. A similar two

component hypothesis for pancreatic calcium secretion has been formulated independently by GOEBELL, STEFFAN & BODE (1972) in a recent *in vivo* study of calcium secretion in the pancreatic juice of the dog.

3. Effects of calcium, magnesium and barium on enzyme secretion

Following the early studies of DOUGLAS and his coworkers on the secretion of catecholamines from perfused adrenal glands (DOUGLAS, 1968) a dominant role has been established for calcium ions in the exocytotic secretion of many different macromolecules from a variety of neural, endocrine and exocrine tissues (see RUBIN, 1970). It is thought that an influx of extracellular calcium ions acts as the coupling agent between the stimulus and the secretory mechanism, a process termed 'stimulus-secretion coupling' by analogy to the mechanism of muscle contraction. (DOUGLAS, 1968).

Calcium removal had little immediate effect on amylase secretion from the *in vitro* cat pancreas but prolonged perfusion with calcium free fluids containing EGTA reduced basal amylase secretion and reduced acetylcholine and CCK-PZ stimulated secretion. This effect was not due to EGTA alone since its addition to perfusates of normal calcium concentration caused no inhibition of enzyme secretion. Similar actions of calcium free media on amylase secretion have been observed in other *in vitro* preparations of pancreas (HOKIN 1966, ROBBERECHT & CHRISTOPHE, 1971; CASE & CLAUSEN, 1971 a & b) and in parotid gland (RASMUSSEN & TENENHOUSE, 1968; SELINGER & NAIM, 1970) and submaxillary gland (DOUGLAS & POISNER, 1963). However evidence obtained from depletion studies does not necessarily mean that calcium ions are involved in the mechanism of the secretory process. These

results could be explained in terms of the many other effects that calcium is known to have on cellular function (RUBIN, 1970). This view is supported by the fact that calcium free buffers reduce the resting potential of pancreatic acinar cells by 25% (PETERSEN & MATTHEWS, 1972). In addition substantial effects of calcium depletion were slow to develop and only partially reversible suggesting that prolonged exposure to calcium-free solutions causes damage to the acinar cells.

Alterations in perfusate magnesium concentration had no marked effects on amylase secretion from the isolated cat pancreas. Similar observations have been made by KANNO (1972). Only weak inhibitory effects of excess magnesium have been demonstrated in the fragmented rat pancreas (ROBBERECHT & CHRISTOPHE, 1971) and the cat submaxillary gland (DOUGLAS & POISNER, 1963). In agreement with ROBBERECHT & CHRISTOPHE (1971) barium did not substitute for calcium in maintaining enzyme secretion from the pancreas.

These results differ somewhat from those previously described for nerve terminals and some endocrine tissues in which the secretory product is packaged in granular form. Thus the secretion of catecholamines and acetylcholine from nerve terminals, catecholamines from the adrenal medulla, vasopressin and oxytocin from the neurohypophysis; T.S.H., L.H., ACTH and prolactin from the adenohypophysis; and insulin from the pancreatic β cell are all rapidly inhibited by reduction in the calcium and elevation of the magnesium concentrations in the bathing media (RUBIN, 1970). In all these tissues barium and or strontium act as agonists of extracellular calcium.

It is therefore concluded that in contrast to their established role in some other secretory processes extracellular calcium ions do

not play a very important role in hormorally stimulated enzyme secretion from the exocrine pancreas. The observations of PETERSEN & N ISHIYAMA (1974) and NISHIYAMA & PETERSEN (1974) that the primary action of acetylcholine on rat pancreas acinar cells is to increase the membrane permeability to sodium and potassium ions and that there is no electrophysiological evidence for an influx of calcium ions add weight to this conclusion. These results thus raise two important questions. Is an elevated cytosolic concentration of calcium ions an activating step in the secretory processes of the pancreatic acinar cell and if this is so from where is this calcium derived.

The answer to the first question is probably yes since a number of manoeuvres which increase the influx of calcium ions into the acinar cell also stimulate enzyme secretion. Elevating the perfusate calcium concentration from 2.5 - 10.0 m Eq/l caused only a small transient release of amylase but repeating this during submaximal acetylcholine infusion elicited a large additional response. This effect was not due to the release of acetylcholine from cholinergic nerve terminals within the gland since it also occurred during submaximal CCK-P% stimulation in the presence of atropine. A similar observation has been reported for the perfused cat submaxillary gland but appears to be secondary to the release of transmitter substances (DOUGLAS & POISNER, 1963). SCHULZ & MANNIGEL (1974) observed that single injections of 0.1 - 1.0 ml isotonic calcium chloride caused, in the presence of atropine, a dose dependent protein and amylase secretion from the perfused cat pancreas. This effect was increased 50-100% in the presence of a low dose infusion of acetylcholine. Increasing the permeability of the plasma membrane to calcium ions by

use of the ionophore A23187, a condition which leads to a measurable increase in calcium uptake by the gland also stimulates amylase secretion (SELINGER, PIMERL, SAVION & SCHRAMM, 1974; WILLIAMS & LEE, 1974). Furthermore CASE & CLAUSEN (1973) have shown that removal of extracellular sodium ions, a process which leads to a reduction in calcium efflux and an increase in calcium influx in nerve and muscle causes an atropine resistant secretion of amylase from the uncinate pancreas of baby rats. Unlike hormone stimulated amylase secretion the effect of sodium lack was abolished by reducing the extracellular calcium concentration to 0.1 mM and was associated with an increase in the uptake of ^{45}Ca from the bathing media. Taken together these results suggest that an increase in the cytosolic concentration of calcium ions is indeed an important step in the process of enzyme secretion from the exocrine pancreas, and that under certain 'abnormal' circumstances these calcium ions may be derived from an extracellular site.

Thus if calcium is a link in the stimulus-secretion coupling process the possibility arises that physiological stimulants of enzyme secretion release calcium from an intracellular store. CASE & CLAUSEN (1973) demonstrated that acetylcholine and CCK-PZ have no effect on ^{45}Ca uptake in the baby rat pancreas but cause a dose dependent ^{45}Ca efflux from the preloaded gland. Similar results have been reported for the parotid (DREISBACH 1964) and submaxillary salivary glands (NEILSEN & PETERSEN, 1972). Conversely the adrenal medulla (RUBIN, FEINSTEIN, JAANUS & PALMRE, 1967) and pancreatic islets (MALAISSE-LEGAE & MALAISSE, 1971) show a dose dependent uptake of ^{45}Ca in response to secretory stimulants. The analogy with excitation-contraction coupling may thus be extended by concluding that nerve

terminals and endocrine cells behave like smooth muscle deriving their activating calcium from an extracellular site whereas exocrine cells are more like striated muscle deriving calcium from an intracellular store.

The site of this intracellular calcium store is as yet undefined, but presumably exists as an actively accumulated store within a 'sarcoplasmic like' reticulum, the mitochondria or the zymogen granules or as calcium passively bound to intracellular membranes. Subcellular fractionation of the rat pancreas has revealed that nucleotide triphosphate [illegible] stimulated accumulation of calcium ions is solely a property of the mitochondria (ARGENT, SMITH & CASE, 1975). Microsomal calcium pumps similar to those described in rat parotid (SELINGER, NAIME & LASSER, 1970) and submaxillary salivary glands (SELINGER et al 1970; ALONSO , BAZERQUE, ARRIGO & TUMILASCI, 1971) were not detected.

4. Effects of sodium and potassium on enzyme secretion.

The action of potassium in releasing amylase from the pancreatic acinar cell was not unexpected as many precedents exist for the liberation of both hormones and transmitter substances by potassium. The effects of rapid intra-arterial injections of potassium chloride in causing the secretion of adrenaline from the adrenal medulla were reviewed as early as 1940 by FENN. Since then the number of hormones shown to be released by high potassium concentrations in the extracellular fluid has steadily increased: vasopressin (DOUGLAS & POISNER, 1964), thyrotropin and adrenocorticotrophic hormone (VALE & GUILLEMIN, 1967), luteinizing hormone (SAMLI & GESCHWIND, 1968), follicle stimulating hormone (JUTISZ & PALAMO de la LLOSA, 1970) and growth hormone (MACLEOD & FONTHAM, 1970). Elevated potassium con-

centrations also stimulate the release of insulin from both perfused (GRODSKY & BENNETT, 1966) and cultured (LAMBERT, JEANRENAUD, JUNOD & RENOLD, 1969) rat pancreas and from rabbit pancreas *in vitro* (HALFS & MILNER, 1968). Potassium ions also have a stimulatory action on exocrine glands. BDOLAH, BEN-ZVI & SCHRAMM (1964) have reported that amylase secretion is stimulated from rat parotid gland slices when the concentration of potassium is raised to 20 - 60 mM, conditions which also cause in increase in the concentration of cyclic 3', 5'-adenosine monophosphate in this tissue (RASMUSSEN & TENENHOUSE, 1968).

The results presented in this thesis demonstrate that perfusing the isolated cat pancreas with potassium-rich solutions stimulates the secretion of amylase. Quantitatively the 'dose-response' curve for potassium-stimulated amylase secretion is similar to that obtained by DOUGLAS & POISNER (1964) for the release of vasopressin from the rat neurohypophysis. The effect of atropine and the detection of acetylcholine in the effluent from the gland, suggest that the stimulatory effect of potassium is an indirect one secondary to the release of acetylcholine.

As early as 1936 BROWN & FELDBERG demonstrated that potassium and to a lesser extent caesium would release acetylcholine from the perfused superior cervical ganglion of the cat. Furthermore FELDBERG & GUIMARAIS (1936) observed the release of acetylcholine by potassium, but not caesium, from the submaxillary gland of both cat and dog and from the sweat glands and tongue of the cat. Acetylcholine also appeared in the effluent from the perfused frog heart when Ringer's solution containing excess potassium was used (BERNAK, 1934). Confirmation of the action of potassium on nervous tissue came when MANN, TENNENBAUM & QUASTEL (1939) demonstrated that potassium, rubidium

and to a lesser extent caesium released acetylcholine from respiring brain slices. The finding that caesium was more effective in stimulating amylase secretion from the isolated pancreas than either potassium or rubidium is difficult to reconcile with these early papers in which caesium was usually less effective in liberating acetylcholine.

Acetylcholine has been assumed to be the transmitter substance responsible for enzyme secretion by the pancreas as a result of anatomical studies (COUPLAND, 1958) and the effects of atropine (BROWN, HARPER & SCRATCHERD, 1967) but it has not been identified in the blood or perfusate issuing from the pancreas after vagal stimulation. The action of potassium reported in this paper seems to be due to release of acetylcholine from vagal postganglionic nerve terminals, thus providing further evidence for acetylcholine being the transmitter substance. However potassium would be expected to stimulate preganglionic nerve terminals as well as postganglionic nerves. Evidence that the liberation of amylase depends on the action of potassium on postganglionic nerves was obtained by using hexamethonium to block ganglionic transmission. In the intact cat hexamethonium blocks the secretion of amylase in response to vagal stimulation (BROWN et al., 1967) whereas the addition of hexamethonium bromide (10 mg/l) to the perfusate of the isolated gland had no effect on the amylase response to potassium.

Since potassium indirectly stimulates the secretion of amylase from the perfused cat pancreas the question must be raised as to whether potassium has an indirect action on other secretory tissue. The observations that potassium has a direct effect on the chromaffin cells of the adrenal medulla (VOGT, 1952; DOUGLAS & RUBIN, 1961) may

also apply to the *in vitro* rat neurohypophysis since neither acetylcholine, carbachol nor eserine stimulates the release of vasopressin (DOUGLAS & POISNER, 1964). However SCHRAMM (1968) has shown that potassium ions stimulate amylase secretion from rat parotid gland indirectly, by liberating stores of endogenous catecholamines.

These results suggest that secretory tissues of neural origin viz. nerve terminals, sympathetic ganglia, the neurohypophysis and adrenal medulla are all directly stimulated by potassium whereas other secretory tissues are not. However a number of observations i.e. the effects of potassium on insulin secretion from the β cell and T.S.H., L.H. and F.S.H. secretion from the adenohypophysis present themselves as apparent exceptions to this general rule. It is possible that the reported effect of potassium on insulin secretion is secondary to the release of acetylcholine since stimulation of the vagal supply to the perfused rabbit pancreas increases the rate of insulin secretion from the gland (FINDLAY, GILL, LEVER, RANDLE & SPRIGGS, 1969). Also, in isolated rat pancreatic tissue acetylcholine potentiates insulin secretion stimulated by glucose (MALAISSE, MALAISSE-LAGAE, WRIGHT & ASHMORE, 1967). Furthermore none of the studies on hormone release from the adenohypophysis have included the effects of neurotransmitter substances and blocking agents. A more likely explanation however is that at least two of these apparently non-nervous secretory cells are in fact of neural origin. Both the pancreatic β cell (insulin) and the corticotroph cell (ACTH) of the anterior pituitary have been classified as members of the A.P.U.D. cell series (amine content and/or amine precursor uptake and decarboxylation) (PEARSE & WELBOURNE, 1973) and are derived phylogenetically from the neural crest.

Potassium free fluids probably also act in an indirect manner to increase basal amylase and protein secretion from the perfused cat pancreas since this effect was blocked by atropine. Potassium deprivation increases the spontaneous release of acetylcholine from motor nerve terminals (BIRKS & COHEN, 1968).

Neither potassium excess nor deprivation influenced the stimulatory effect of CCK-PZ or acetylcholine on the acinar cells. Thus the small depolarization of acinar cells due to acetylcholine and CCK-PZ (DEAN & MATTHEWS, 1972; PETERSEN & MATTHEWS, 1972) although reflecting an altered membrane permeability to sodium and potassium ions (PETERSEN & MATTHEWS, 1972) is presumably not a sine qua non for enzyme secretion. Similar conclusions have been reached with regard to the actions of acetylcholine on the adrenal medulla (DOUGLAS & RUBIN, 1963; DOUGLAS, KANNO & SAMPSON, 1967) and ACTH on the adrenal cortex (MATTHEWS & SAFFRAN, 1967; JAANUS, ROSENSTEIN & RUBIN, 1970).

Reducing the concentration of extracellular sodium to 50 mM had no effect on the enzyme secretory response to acetylcholine. Experiments involving total sodium replacement were subject to technical difficulties (outlined in Results) arising from the inhibition of electrolyte secretion and viscosity of the substituent sucrose. They demonstrated no consistent effect of sodium lack on acetylcholine stimulated amylase secretion. RIDDERSTAP & BONTING (1969) have also shown that basal enzyme secretion from the isolated rabbit pancreas is unaffected during prolonged exposure to a bathing fluid containing 25 mM/l sodium. However, in contrast, CASE & CLAUSEN (1973) have reported that 90 mins after replacement of extracellular sodium by lithium the secretion of amylase by the baby rat pancreas in response to CCK-PZ is abolished. If an increase in the intracellular con-

centration of sodium ions were an important link in stimulus secretion coupling one would predict that the inhibition of Na/K ATPase with ouabain would stimulate enzyme secretion. This was not however found to be the case (CASE & CLAUSEN, 1973). Obviously the role of sodium distribution across the acinar cell plasma membrane in relation to enzyme secretion requires further clarification.

5. Effects of sodium, potassium, magnesium and calcium on electrolyte secretion.

Magnesium-free, magnesium-rich or calcium-rich perfusates did not havemarked effects on the rate of secretin stimulated electrolyte secretion. The inhibitory effects of reducing the extracellular sodium and potassium concentrations have been described previously (CASE et al., 1968; CASE, et al., 1969b).

Besides stimulating amylase secretion excess potassium decreased both the rate of perfusion and the volume of pancreatic juice secreted in response to secretin. The fall in perfusion rate was presumably due to vasoconstriction as was observed by BROWN & FELDBERG (1936) in the perfused superior cervical ganglia. In these experiments the vasoconstriction was probably partly due to the liberation of catecholamines from sympathetic nerve terminals since it was substantially decreased after α -receptor blockade. Furthermore, the potassium-rich effluent contained more pressor activity than could be accounted for by its potassium content when tested on the atropinized eviscerated cat. The decrease in secretion rate was not secondary to the decreased perfusion rate since decreasing the flow of normal perfusate to levels observed during potassium stimulation had little or no effect on secretory rate. Similarly when lithium was tested in

place of potassium the reduction in secretory rate persisted in the absence of any effect on perfusate flow rate. It seems more likely that the reduction in secretory rate can be explained by the decrease in sodium concentration of the perfusion fluid since it is known that the volume of pancreatic secretion is reduced under these conditions (CASE et al., 1968). If sodium lack alone is responsible for the reduction in secretory rate the effect of replacing sodium by potassium ions should be approximately equal to that obtained using sucrose. This was found to be so.

The inhibitory effect of calcium-free media on pancreatic electrolyte secretion, which has not previously been described, was slower to develop than that on enzyme secretion. A similar situation exists in the submaxillary gland (DOUGLAS & POISNER, 1963). This suggests a different sensitivity of the two secretory processes to calcium. This delayed inhibitory effect is in marked contrast to the immediate effects produced by removal of sodium, potassium or bicarbonate in pancreas (CASE et al. 1969b; CASE, SCRATCHERD & WYNNE, 1970) or of sodium in the submaxillary gland (MARTINEZ & PETERSEN, 1972).

A calcium requirement for gastric acid secretion has also been demonstrated (FORTE & NAUSS, 1963; JACOBSON, SCHWARTZ & REHM, 1965). The cause of inhibition in these and other electrolyte transport processes is difficult to assess. It may result from alterations in the permeability of the cell membrane (MANERY, 1966); or of the junctional complex between cells, which apparently acts as the principal route of passive ion permeation in gall bladder, and perhaps other tissues (DIAMOND, BARRY & WRIGHT, 1971). Certainly EDTA treatment causes increased movement of sucrose across bullfrog gastric mucosa (FORTE & NAUSS, 1963) by loosening the junctional complex between

gastric cells (SEDAR & FORTE, 1964) and a similar explanation may account for the increased calcium concentration in pancreatic juice collected immediately after a prolonged period of calcium-free perfusion. Whether calcium ions have a direct effect on the electrolyte secretory mechanism remains to be determined.

6. The effects of tetracaine on enzyme and electrolyte secretion.

The local anaesthetic tetracaine is known to inhibit catecholamine secretion from the chromaffin cells of the adrenal medulla by blocking the influx of calcium ions that occurs in response to acetylcholine stimulation (DOUGLAS & KANNO, 1967). It has similar effects on pancreatic amylase secretion at a concentration that was without effect on electrolyte secretion. This would seem to provide evidence for a role of extracellular calcium ions in the secretory process. However tetracaine is also antagonistic to the action of acetylcholine (RUBIN, et al., 1967) and this may explain its effects on the pancreas. In addition tetracaine reduces the depolarizing effect of acetylcholine and CCK-PZ on single acinar cells (MATTHEWS & PETERSEN, 1973) suggesting that it may owe its effect to an interference with sodium influx since this is the only inward current carrier (PETERSEN & NISHIYAMA 1974).

7. The effects of colchicine on enzyme and electrolyte secretion.

The effects of the alkaloid colchicine on mitotic spindle fibres and a number of other intracellular systems concerned with cell structure and movement have been ascribed to the dissolution of microtubules (see OLMSTED & BORISY, 1973). These actions are associated with the binding of colchicine to a protein dimer of

molecular weight 110,000 which forms a subunit of the microtubule. The observation that the insulin containing granules of the β cell are arranged on microtubular like structures (ORCI, LIKE, AMHERDT, BLONDEL, KANAZAWA, MARLISS, LAMBERT, WOLLHEIM & RENOLD, 1973) provides a morphological basis for the fact that colchicine has inhibitory effects on insulin secretion from isolated islets of Langerhans (LACY, HOWELL, YOUNG & FINK 1968). Similar inhibitory effects of colchicine on thyroid secretion have been reported (WILLIAMS & WOLFF, 1970). In one of two experiments in which colchicine was tested on the perfused cat pancreas a partially reversible inhibition of acetylcholine stimulated amylase secretion was detected. As yet no convincing morphological or biochemical evidence has been presented for the occurrence of microtubules in the exocrine pancreas so the significance of this effect remains uncertain. In both experiments colchicine caused a progressive increase in the rate of electrolyte secretion stimulated by supra-maximal secretin infusion. The reason for this remains obscure.

8. <u>The effects of nystatin on enzyme and electrolyte secretion.</u>

Certain polyene antibiotics e.g. nystatin increase the permeability of artificial lipid bilayers to calcium and other divalent ions (VAN ZUTPHEN, 1970). Similar effects have been observed in biological membranes of high sterol content such as giant squid axons where external application of nystatin in low concentrations increases the intracellular ionized calcium level (CRAWFORD & FETTIPLACE, 1971). Predicting that a similar action of nystatin on pancreatic acinar cells would lead to enzyme secretion one experiment was performed in which the drug was perfused through the gland at a

concentration of 1.5×10^{-4}M. The result was a progressive reduction in electrolyte secretion and a 50% inhibition of acetylcholine stimulated amylase secretion. CRAWFORD & FETTIPLACE (1971)noted that prolonged exposure of their squid axons to nystatin resulted in a steady deterioration of the preparation. This could be the cause of the inhibitory effects noted in the pancreas especially in view of the high concentration employed.

In conclusion the results presented in this section suggest that unlike some other secretory tissues extracellular calcium ions may not play an important role in pancreatic enzyme secretion but are probably involved in the general maintenance of glandular function. However, evidence from other sources suggests that an increase in the cytosolic concentration of calcium ions fulfills an important function in the enzyme secretory mechanism and that this calcium is derived from an intracellular store. Alterations in the concentrations of other extracellular cations Na^+, K^+ and Mg^{++} appear to have no direct effect on the functioning of the acinar cell although elevated concentrations of potassium stimulate amylase secretion by releasing acetylcholine from nerve terminals within the gland. Exactly how the ubiquitous calcium ion acts as a trigger for enzyme secretion remains a mystery.

PART II

ISOLATED PANCREATIC ACINAR CELLS

INTRODUCTION

The use of isolated cells in suspension for the study of cellular function offers a number of advantages. The system is simple and can be manipulated with ease. It provides for a faster and more complete contact of the entire cell population with compounds present or added to the incubation medium - a condition not always fulfilled by other *in vitro* systems e.g. tissue slices. Suspensions of cells may be drawn evenly from different parts of an organ thus giving a high degree of experimental uniformity. Metabolic and morphological studies can be easily combined, and it allows a study of the functional characteristics of a particular cell in a heterogeneous tissue, provided that cell can be isolated as a pure population.

Experience has shown that all isolation techniques, no matter how carefully designed, result in varying degrees of morphological and or biochemical damage to the cells in question. The possibility should also be considered that the resultant single cells, deprived of a close physical association with their neighbours may undergo subtle and perhaps unrecognised alterations in their characteristics. All epithelial cells are electrically coupled (LOEWENSTEIN, SOCOLAR, HIGASHINO, KANNO & DAVIDSON, 1965) and thus perhaps metabolically coupled *in situ*. Thus the concept of a tissue as a collection of individual units which can influence or be influenced only by extracellular pathways may be erroneous. In addition some cells, e.g. exocrine secretory cells, exhibit a structural polarity *in situ* which means that different areas of the plasma membrane are exposed to extracellular fluids of vastly different composition. This polarity is unavoidably lost to cells in suspension.

Thus the experimenter in his quest for simplification may be guilty of making observations on a grossly unphysiological model. Nevertheless it has been said that the science of biochemistry is based on the work of individuals brave enough to break up cells and draw physiological conclusions from their results. Time may award the cellular physiologist a similar honour. Bearing in mind the possible shortcomings and provided that the use of isolated cells offers technical advantages over other in vitro systems then this approach can be justified.

Ideally any technique for the isolation of single cells from a solid tissue should fulfill three criteria:-

1. It should result in individual cells in good physical, biochemical and structural condition.
2. The method should provide a high yield.
3. The final suspension should be free of blood cell elements and cellular debris.

In practice the final method always represents a compromise usually arrived at by trial and error rather than by predictive thought. Most trouble is presented by the balance between the first and second criteria since a few cells in good condition are as worthless, for biochemical studies at least, as a large number of damaged ones.

In the past a number of general tissue dissociation techniques have emerged. These can be classified as follows:-

1. Methods involving mechanical dissociation of the tissue.
2. Methods in which the tissue is perfused, sometimes with a calcium chelating agent, and then dispersed with mechanical force.

3. Methods in which the intercellular cement is softened or removed by a chemical or a physical treatment not involving perfusion.

4. Methods based on the enzymic treatment of the tissue to degrade a component of the intercellular cement. Often this technique is combined with the use of a calcium chelating agent.

Each of these methods will now be discussed in more detail. Although in recent years isolated cells have been successfully obtained from a wide variety of tissues most of the pioneering studies utilized the liver.

1. Mechanical Dispersion

All of the early attempts to obtain isolated cells from the liver employed a variation of this general approach. The earliest recorded is that of BOHM (1931) who managed to isolate hepatocytes from a number of species by chopping, teasing or macerating the tissue between glass slides. His interests were solely morphological and it was more than a decade before the biochemical study of single cells began in earnest. SCHNEIDER & POTTER (1943) isolated liver cells by forcing the tissue through cheesecloth followed by bolting cloth. A similar technique employing stainless steel screens of increasingly fine mesh was used by KALTENBACH (1952). Mild degrees of homogenisation more usually employed to obtain a totally disrupted tissue have also been used with some success. (ELLIOT & LIBET, 1942; PALADE & CLAUDE, 1949). Shaking liver slices with glass beads either with (HENLEY, SORENSEN & POLLARD, 1959) or without (St. AUBIN & BUCHER, 1952) a calcium chelating agent has also proved effective. The disadvantages of these early methods were that the cells were exposed to excessive

stress, yields were low (usually less than 10%) and the final suspensions were often contaminated with blood cell elements (ANDERSON, 1953; LAWS & STICKLAND, 1956).

2. Perfusion plus mechanical dispersion.

The combination of perfusion and mechanical techniques offered a number of advantages. Contamination with blood cells was considerably lower and inclusion of a calcium chelating agent in the perfusion fluid reduced the degree of mechanical force subsequently required to dissociate the tissue. ANDERSON (1953) was the first worker to develop this approach and it can be fairly said that his efforts mark the point at which the biochemical study of isolated cells began to gain momentum. His technique required the liver to be perfused with a calcium free Locke's solution containing citrate, pyrophosphate, EDTA, ATP or glycerophosphate and the blanched tissue to be gently homogenised employing a loose fitting pestle. The claimed 50% yield represented a marked improvement over earlier methods but was not confirmed by later workers, their recoveries being nearer 20%. (LAWS & STICKLAND, 1956; JACOB & BHARGAVA, 1962). Nevertheless this technique was a significant advance over those previously available and has seen wide application, sometimes with slight modifications. (LAWS & STICKLAND, 1956; LATA & REINERTSON, 1957; KALANT & YOUNG, 1957; BASS & SALTMAN, 1959; BEN-OR & DOLJANSKI, 1960; ZIMMERMAN, DEVLIN & PRUSS, 1960; GIBBONS & RIENITZ, 1961; JACOB & BHARGAVA, 1962).

In some instances perfusion has been performed without a chelating agent in an attempt to minimise possible deleterious effects of these compounds. The obvious disadvantage is that extra mechanical

force is required to dissociate the tissue. BRANSTER & MORTON (1957) utilised a variety of perfusing agents including Locke's solution, sucrose of different tonicity, magnesium chloride and polyvinyl pyrrolidone. The final result however appeared independent of the perfusing agent used. Tissue dispersion was performed with an homogeniser and although these workers did not provide data on yields the same technique in the hands of BERRY (1962) gave surprisingly high recoveries of 40-80%.

3. Softening of intercellular materials by chemical or physical means without the use of perfusion.

This approach is exemplified by the method of LONGMUIR & REES (1956). Liver cells were dissociated from slices by incubation in phosphate buffered saline at low pH (5.0) followed by repeated pipetting. Lowering the pH of the medium increased the yield. This method necessitates the cells being subjected to a non-physiological hydrogen ion concentration.

4. Enzymic digestion of intercellular materials.

Although the use of trypsin to dissociate cells in tissue culture was advocated by ROUSE & JONES (1916) almost four decades elapsed before this technique was applied to solid tissues. (MOSCONA & MOSCONA, 1952). Since then it has seen extensive and recently almost exclusive use being applied to a wide variety of tissues from a number of different species. This approach is favoured for its capacity to produce high yields of cells without the need for vigorous physical procedures, although some degree of mechanical disruption is always retained. Ideally the enzyme(s) chosen should do minimal

damage to the cells and be active in an ionic environment compatible with cell survival.

Some idea of the wide applicability that this technique has gained can be had from table II which lists the permutations of tissues and enzymes which have proved successful. Although RINALDINI (1958) in an excellent review has attempted to elevate the enzymic digestion of intercellular cements to a philosophical level much of it remains empirical. This is borne out by the fact that a number of different enzymes have been used to isolate cells from the same tissue (see Table II). In addition crude preparations of the enzymes usually employed for tissue dissociation often contain activities other than those stated on the label of the bottle, eg commercial preparations of collagenase contain variable levels of non-specific proteases (AMSTERDAM & JAMIESON, 1972). These extra 'activities' may be critically important in providing effective tissue dissagregation (AMSTERDAM & JAMIESON, 1972) and can lead to situations where different batches of the same enzyme are ineffective (KLOPPENBERG, ISLAND, LIDDLE, MICHELAKIS & NICHOLSON, 1968).

5. The viability of isolated cells.

Having discussed the various methods available for the isolation of single cells the next question to answer is how closely do they resemble their fellows in situ. Previous workers have attempted to answer this question from the point of view of fine structure, respiratory activity and biochemical integrity. Again what follows is mainly derived from observations on isolated liver cells since these have been most thoroughly studied.

Studies on the structure of cells isolated by methods involving

TABLE II The isolation of single cells from a variety of tissues by enzymic procedures.

TISSUE	ENZYMES	CONDITIONS	REFERENCE
LIVER	1) TRYPSIN	INCUBATION	DULBECCO,R.& VOGT,M. (1954) J. Exp. Med. 99 167.
	2) COLLAGENASE - HYALURONIDASE	PERFUSION Ca, Mg free EDTA	BERRY,M.N. & FRIEND,D.S. (1969) J. Cell. Biol. 43 506-502.
	3) COLLAGENASE HYALURONIDASE	PERFUSION Ca free	HOWARD,R.B., CHRISTENSEN,A.K., GIBBS, F.A.I. & PESCH,L.A. (1967) J. Cell Biol. 35 675-684.
	4) LYSOZYME	PERFUSION EDTA	HOMMES,F.A., DRAISMA,M.I. & MOLENAAR,I. (1970) Biochim., Biophys. Acta. 222 361-371.
ADRENAL CORTEX	1) COLLAGENASE	INCUBATION	KLOPPENBORG,P.W.C., ISLAND,D.P., LIDDLE,G.W., MICHELAKIS,A.M. & NICHOLSON,W.D. (1968) Endocrinology. 82 1053-1058.
	2) TRYPSIN	INCUBATION	SAYERS,G., SWALLOW,R.C., GIORDANO,N.D. (1971) Endocrinology. 88 1063-1068.
FAT PADS	1) COLLAGENASE	INCUBATION	RODBELL M. (1964) J. Biol. Chem. 239 375-380.

TISSUE	ENZYMES	CONDITIONS	REFERENCE
SMALL INTESTINE	1) HYALUDRONIDASE	INCUBATION	KIMMICH, G.A. (1970) Biochemistry. 9 3659-3667.
	2) HYALURONIDASE	INCUBATION	PERRIS, A.D. (1966) Can. J. Biochem. 44 687-693.
	3) TRYPSIN PANCREATIN	INCUBATION	HARRER, D.S., STERN, B.K. & REILLY, R.W. (1964) Nature. 203 319-320.
OVARIAN TISSUE	1) TRYPSIN COLLAGENASE	INCUBATION	LIU, T.C. & GORSKI, J. (1971) Endocrinology. 88 419-426.
CORPUS LUTEUM	1) COLLAGENASE HYALURONIDASE	INCUBATION	GOSPODAROWICZ, D & GOSPODAROWICZ, F. (1972) Endocrinology. 90 1427-1434.
LUNG	1) COLLAGENASE DNase	PERFUSION then INCUBATION Ca free	AYUSO, M.S., FISHER, A.B., PARILLA, R. & WILLIAMSON, J.R. (1973) Am. J. Physiol. 225 1153-1160.
GASTRIC MUCOSA (PARIETAL CELLS)	1) COLLAGENASE	INCUBATION	WALPER, A.I. & LUNSETH, J.B. (1963) Proc. Soc. Exp. Biol. Med. 112 494-496.

TISSUE	ENZYME	CONDITIONS	REFERENCE
GASTRIC MUCOSA (PARIETAL CELLS)	2) PRONASE	INCUBATION	BLUM, A.L., SHAH, G.T., WIEBELHAUS, V.D., BRENNAN, F.T. HELANDER, H.F., CEBALLOS, R. & SACHS, G. (1971) Gastroenterology. 61 189-200.
	3) COLLAGENASE PRONASE	INCUBATION	CROFT, D.N. & INGELFINGER, F.T. (1969) Clin. Sci. 37 491-501.
SOLID TUMORS	1) TRYPSIN DEOXYRIBONUCLEASE	INCUBATION	MADDEN, R.E. & BURK, D.J. (1961) J. Natl. Canc. Inst. 27 841-855.
EMBRYONIC HEART	1) COLLAGENASE	INCUBATION Ca & Mg free	GUIDOTTI, G.C., LUNEBERG, B., & BORGHETTI, A.F. (1969) Biochem. J. 114 97-105.
HEART	1) COLLAGENASE	INCUBATION	CAVANAUGH, D.J., BERNDT, W.D. & SMITH, T.E. (1963) Nature 200 261-262.
KIDNEY	1) TRYPSIN	INCUBATION	DULBECCO, R. & VOGT, M. (1954) J. Exp. Med. 99 167.
TESTIS	1) TRYPSIN	INCUBATION	DULBECCO, R. & VOGT, M. (1954) J. Exp. Med. 99 167.
ANTERIOR PITUITARY	1) TRYPSIN	INCUBATION	PORTANOVA, R., SMITH, D.K. & SAYERS, G. (1970) Proc. Soc. Exp. Biol. Med. 133 573-576.

TISSUE	ENZYME	CONDITIONS	REFERENCE
MAMMARY GLAND	1) COLLAGENASE HYALURONIDASE PRONASE	INCUBATION Ca, Mg free	WIEPSES, J.A. & PROP, F.J.A. (1970) Expl. Cell Res. 61 451-454.
PANCREAS	1) COLLAGENASE HYALURONIDASE	INCUBATION Ca, Mg free EDTA	AMSTERDAM, A. & JAMIESON, J.D. (1972) Proc. Natl. Acad. Sci. U.S.A. 69 3028-3032.
	2) COLLAGENASE TRYPSIN HYALURONIDASE	INCUBATION Ca, Mg free EDTA	AMSTERDAM, A. & JAMIESON, J.D. (1974) J. Cell. Biol. 63 1037-1056.
THYROID	1) TRYPSIN DEOXYRIBONUCLEASE	INCUBATION	MAAYAN, M.L. MILLER, S.L. & INGBAR, S.H. (1971) Endocrinology 88 620-626.
	2) TRYPSIN DEOXYRIBONUCLEASE	INCUBATION	TONG, W. (1964) Endocrinology. 74 304-306
	3) TRYPSIN	INCUBATION	TONG, W., KERKOF, P. & CHAIKOFF, I.L. (1962) Biochim. Biophys. Acta. 60 1-19.
AORTIC ENDOTHELIAL CELLS	1) TRYPSIN	INCUBATION	POMERAT, C.M. & SLICK, W.C. (1963) Nature. 198 859-861.

vigorous mechanical procedures are sadly lacking. BERRY & SIMPSON (1962), EXTON (1964) and GWYNN, JONES, JONES & KEMP (1970) have reported that such cells show extensive structural changes as compared to the intact liver including partial disruption of the plasma membrane, vacuolation of the cytoplasm, vesicular distortion and contraction of the mitochondria.

These structural changes are paralleled by abnormalities at the biochemical level. Of particular importance is the loss of soluble components, especially enzymes, from the cytoplasm. HENLEY et al. (1959); BERRY (1962); EXTON (1964) and TAKEDA, ICHIHARA, TANIOKA & INOUE (1964) have reported the loss of most of the cellular content of glutamate pyruvate transaminase, lactate dehydrogenase, isocitric dehydrogenase, glucose-6-phosphate dehydrogenase, aldolase, L-iditol dehydratase, tryptophan-pyrrolase, serine and threonine dehydratases and nicotinamide nucleotides from liver cells. Particulate enzymes such as glucose-6-phosphatase and enzymes of the tricarboxylic acid cycle were retained. Most of the glycogen is also lost from these cells. (KALANT & YOUNG, 1957; EXTON, 1964). Much of this damage probably resulted from the mechanical treatment used to disrupt the tissue since TAKEDA et al. (1964) observed that perfusion of the liver, a frequent preliminary to mechanical disruption, did not result in enzyme leakage.

As might be expected alterations in the respiratory characteristics of the isolated cells have also been observed. A number of studies have shown that mechanically isolated hepatocytes possess endogenous respiratory activity (LONGMUIR & ApREES, 1956; BERRY, 1962; EXTON, 1964; IYPE & BHARGAVA, 1965; ONTKO, 1967). In one study the rate of endogenous respiration was similar to that of liver

slices (LONGMUIR & Ap REES, 1956) whereas IYPE & BHARGAVA (1965) reported it to be a quarter of this value. On the other hand LAWS & STICKLAND (1955) in a comparison of the mechanical methods of ANDERSON (1953), KALTENBACH (1952) and LONGMUIR & Ap REES (1956) were unable to detect endogenous respiration in any of these preparations. Similar results were obtained by ZIMMERMAN et al. (1960) and by KALANT & YOUNG (1957) but may presumably represent substrate deficiencies rather than metabolic damage. Although there appears to be some disagreement over the rate of endogenous respiration in hepatocytes there was general acceptance of the fact that carbohydrate substrates such as glucose, fructose and glucose-1-phosphate do not stimulate it, (LAWS & STICKLAND, 1956; KALANT & YOUNG, 1957; ZIMMERMAN et al., 1960; BERRY, 1962; EXTON, 1964; IYPE & BHARGAVA, 1965), a consequence no doubt of the loss of soluble glycolytic enzymes. ROSE (1967) however has reported that nerve and glial cells mechanically isolated from rat cerebral cortex have rates of glucose stimulated respiration only 20-30% lower than that of intact cortex slices. The final pathways of oxidative metabolism in the particulate mitochondria appeared to be intact since tricarboxylic acid cycle intermediates (KALANT & YOUNG, 1957; LAWS & STICKLAND, 1956; BERRY, 1962; EXTON, 1964; IYPE & BHARGAVA, 1965) and glutamate (BERRY 1962) stimulate respiration and are oxidised by mechanically isolated liver cells. A further indication of mitochondrial function was the fact that hepatocytes oxidise fatty acids (EXTON, 1964) and form urea from ammonium chloride. This intactness of the final pathways of oxidative metabolism may explain the endogenous respiration observed by some workers, the cells acting as if they were a bag of mitochondria. Any uncoupling of electron

transport and oxidative phosphorylation induced by structural damage to the mitochondria, as observed by BERRY (1962), could lead to high rates of endogenous respiration and the erroneous conclusion that the isolated cells were, in this respect at least, comparable to the intact tissue. Interestingly some workers who observed endogenous respiration in isolated hepatocytes have also demonstrated that it was inhibited by increasing the calcium and or phosphate concentration of the extracellular medium (BERRY, 1962; IYPE & BHARGAVA, 1965; ONTKO, 1967). This probably represents an increased permeability of the plasma membrane with consequent penetration of these ions which cause mitochondrial swelling and destruction.

In the light of these observations on early preparations of hepatocytes it is not surprising that EXTON (1964) was forced to conclude that "compared with liver slices rat liver cell suspensions exhibit abnormalities of composition and metabolism that limit their usefulness as an experimental preparation." The recognition that vigorous mechanical treatment was the factor responsible for these abnormalities led to a search for new methods in which this component of the separation procedure was reduced to a minimum. The result of this search was the development of enzymic methods of tissue disaggregation which have subsequently proved so successful. BERRY & FRIEND (1969) have reported that hepatocytes isolated by collagenase and hyaluronidase digestion were structurally normal except for occasional cytoplasmic vesiculation. These authors together with HOWARD, CHRISTENSEN, GIBBS & PESCH (1967) and WIEJPES & PROP (1970) have emphasised that methods involving the least mechanical treatment gave the best results. Chick embryo heart cells (GUIDOTTI, LUNEBERG

& BORGHETTI, 1969) and intestinal epithelial cells (PERRIS, 1966) have also been isolated with good preservation of ultrastructure.

Improvements in function paralleled the improvements in morphology. BERRY & FRIEND (1969) reported that although their hepatocytes lost 60% of their potassium content there was no loss of the cytoplasmic enzyme lactate dehydrogenase. This correlated with the observation that respiration was stimulated by lactate and fructose indicating that much of the catabolic glycolytic pathway was intact - the first demonstration of this fact in an isolated cell. Furthermore these cells synthesised glucose from lactate: a process requiring both mitochondrial and cytoplasmic compartments to work in unison. The advantages of enzyme prepared hepatocytes over those prepared by mechanical means have been effectively summarised by JEZYK & LIBERTI (1969). These authors compared preparations obtained by both methods and found that enzyme prepared cells had a much greater capacity for the incorporation of L-leucine into protein; had 45% higher rates of endogenous respiration; had higher rates of incorporation of palmitate into cholesterol esters (x 2.8), triglycerides (x 3), diglycerides (x 4.3), phosphatidyl ethanolamine (x 21.5) and lecithin (x 14.7); incorporated 20% more uridine into RNA and finally oxidised acetate 6.5 times more effectively.

Single cells retaining many of their <u>in situ</u> characteristics and in some cases physiological response to hormones have been isolated from a number of tissues e.g. fat cells showing insulin stimulated glucose uptake (RODBELL, 1964); thyroid cells incorporating iodine into MIT, DIT, T_3 and T_4 (TONG, 1964); anterior pituitary cells secreting ACTH in response to hypothalamic/median eminence extracts and vasopressin (PORTANOVA, SMITH & SAYERS, 1970); adrenal

cortical cells synthesising corticosterone in response to ACTH (KLOPPENBERG et al. 1968; SWALLOW & SAYERS, 1969; SAYERS, SWALLOW & GIORDANO, 1971); corpus luteum cells secreting progesterone in response to L.H. (GOSPODAROWICZ & GOSPODAROWICZ, 1972); ovarian cells incorporating acetate into steroids in response to L.H. (LIU & GORSKI, 1971); mucosal cells isolated from the small intestine showing active sugar accumulation (KIMMICH, 1970). Such preparations have been used to study receptor function (RODBELL, 1964; KONO, 1969; CUATRECASAS, 1972), to provide simple biological assays for hormones (KLOPPENBERG et al. 1968; SAYERS et al. 1971) for the investigation of transport processes (KIMMICH, 1970) and to act as the starting point for the biochemical study of a particular cell in a heterogeneous tissue (MATEYKO & KOPAC, 1963; AYUSO, FISHER, PARILLA & WILLIAMSON, 1973).

The original aim in applying such techniques to the pancreas was to obtain preparations of duct and centroacinar cells. These are probably the cells concerned with the formation of the electrolyte and water component of pancreatic juice (GROSSMAN & IVY, 1946; de ALMEIDER & GROSSMAN, 1952; KALSER & GROSSMAN, 1954; DREILING et al. 1955; BECKER, 1962). They form a small percentage of the total pancreatic tissue, a fact which precludes study of their biochemistry in whole pancreas homogenates. They would thus seem ideal candidates for isolation - a viable preparation might prove a useful tool with which to investigate pancreatic electrolyte secretory mechanisms. Preliminary experiments indicated however that considerable technical difficulties were involved in separating these cells from the mass of other cell types released by enzymic digestion of the pancreas. Efforts were therefore redirected to the easier

task of obtaining suspensions of acinar cells, an aim which was eventually fulfilled. During the course of an investigation into the biochemical and secretory properties of these cells it became apparent that Drs. Amsterdam and Jamieson working at the Rockefeller University, New York were well advanced on a similar project. To avoid duplication attention was focused on a study of these acinar cells in tissue culture.

This part of the thesis describes the techniques employed for the isolation of acinar cells together with a preliminary investigation of their functional characteristics.

METHODS

A. Preparation of isolated pancreatic acinar cells

1. Physical Disruption of Pancreatic Tissue.

The technique successfully employed by SNEIDER & PERSON (1960), as modified by FLATLAND, SCHNEYER & SCHNEYER (1969) for the isolation of acinar cells from the submaxillary gland was initially tested. Samples of the minced glandular tissue were placed in a sac of nylon sieve cloth, mesh size 153 μ (Tobler, Ernst & Traber New York) and gently compressed, using a glass pestle, in a beaker containing isotonic NaCl (pH 7.4). Submaxillary gland acinar cells were expressed through the sieve cloth by centrifugation. Ductule elements remained in the sac. This method has the advantage that the cells are not exposed to the possible injurious effects of proteolytic enzymes commonly employed in tissue dissociation procedures. It was however unsuccessful in isolating intact acini or acinar cells from the pancreas of guinea-pig and rats, the expressate containing cell debris only.

2. Enzymic Digestion of pancreatic tissue.

Although a number of different enzymes, alone or in combination were tested on the guinea pig pancreas only mixtures of crude collagenase and pronase were effective in isolating single viable acinar cells in sufficient quantities to allow biochemical studies.

Guinea-pigs of either sex weighing approx. 300 g were anaesthetised with ether. The tail of the pancreas was quickly excised through a midline abdominal incision, washed in Hanks

balanced salt solution (Hanks. B.S.S.), trimmed free of fat and mesentery and finely chopped to facilitate oxygen diffusion and penetration of dissociating enzymes into the extracellular spaces. The number of animals used in an experiment varied between two and four. The chopped tissue from each animal was transferred to a 25 ml conical flask containing 10 mls of filtered (Whatman No.1 Filter paper) Eagle's Minimum Essential tissue culture medium (Eagle's M.E.M.), crude collagenase (Koch-Light form I) 50 mg% and pronase (Koch-Light) 50 mg%. After gassing with 95% O_2/5% CO_2 the flasks were incubated at 37°C for 60 mins in a shaking water bath (50 cycles/min), during which time single acinar cells were released from the tissue.

The phenol red indicator contained in the culture medium served as a check that the pH did not change significantly during the incubation. The remaining undigested tissue was drawn through a wide bore pasteur pipette five times. The flask contents were then filtered through nylon sieve cloth (mesh size 153 μ) to remove undigested tissue pieces and the single cells separated from remaining debris by centrifugation (100 g x 5 mins) in capped plastic tubes in an atmosphere of 95% O_2/5% CO_2. The cell pellets were resuspended in a small volume of Eagle's M.E.M. + 5% calf serum, combined, the volume adjusted to 10 ml and sedimented (100 g x 5 mins). This washed cell pellet, diluted to the required volume was used for all further experiments. The whole procedure took about 90 mins.

The proportion of viable cells in the suspension was estimated using an eosin Y dye exclusion test. To a drop of the cell suspension on a slide was added an equal volume of eosin Y at a concentration of 50 mg/100 mls Eagle's M.E.M. After 5 mins the total number of cells and the number of damaged (staining) ones were counted in several

fields. The viability was calculated by the formula:

$$\frac{\text{Total cells - Damaged cells}}{\text{Total cells}} \times 100\%$$

The isolated cells were routinely viewed with a Watson microscope. Micrographs were taken using a Leitz Orthoplan microscope with Heine phase contrast optics.

B. Experiments with Isolated Acinar Cells

1. Incorporation of L-leucine-4-5-H3 into cell protein

This experiment was performed to determine if isolated acinar cells were capable of synthesising protein. The parameter measured was the rate of incorporation of a radioactive amino-acid into trichloroacetic acid (TCA) insoluble material.

A suspension of isolated acinar cells was incubated for 5 hrs in McCoy's 5A medium + 5% calf serum, containing L-leucine-4-5 H3 (Radiochemical Centre Amersham: specific activity approx. 50 Ci/mMol) at a concentration of 2.5 μ Ci/ml. Aliquots of the suspension were withdrawn at the required time intervals, centrifuged (100 g x 10 mins) and the protein-bound radioactivity and total protein content of the cell pellet determined as described below. No correction is made for labelled proteins secreted into the medium during the incubation or for the small amount of calf serum protein that would be associated with the cell pellet.

2. Pulse labelling of isolated acinar cells and the secretion of labelled proteins in response to CCK-PZ, acetylcholine and carbamylcholine

This procedure, based on the pulse labelling techniques developed

by JAMIESON & PALADE (1967a) was used to test the ability of the isolated acinar cells to secrete de novo synthesised protein.

The cell pellet obtained at the end of the isolation procedure was washed once in 10 mls Eagles Basal Culture Medium (B.M.E.) without H1-Leucine. The cells were then pulse labelled for 15 mins at 37°C in 5 mls of the same medium containing 25 μCi L-leucine-4-5-H3. After centrifugation (100 g x 2 mins) the cells were washed once in 10 mls McCoy's 5A medium (0.3 mM L-leucine) + 5% calf serum and then chased in 10 mls of the same medium for 45 mins. This is sufficient time to allow the incorporation of the H3-leucine into secretory proteins and the intracellular transport of these proteins to the zymogen granules (JAMIESON & PALADE, 1967b). The isotope dilution between pulse and chase medium was 3×10^3. The suspension was then divided into two equal fractions and centrifuged (100 g x 5 mins). Each cell pellet was resuspended in 5 mls McCoy's 5A + 5% calf serum and to one was added acetylcholine, carbamylcholine, or CCK-Pz at concentrations specified in the Results, while the other served as a control. Throughout the gas phase was 95% O_2/5% CO_2. After incubation for 2 hrs at 37°C with slow shaking (20 cycles/min) the cells were transferred to conical glass tubes and centrifuged (100 g x 10 mins). The radioactivity associated with the cell pellet and supernatant proteins was determined as described below. Supernatant protein-bound radioactivity is expressed as a percentage of the total protein-bound radioactivity (cells + supernatant).

i.e. $$\frac{\text{Supernatant 3H Protein}}{\text{Supernatant 3H Protein + Cell 3H Protein}} \times 100.$$

3. Extraction and determination of protein.

To a cell pellet resuspended in water or incubation medium was added sufficient ice-cold 50% TCA to give a final concentration of 10%. After mixing and allowing to stand overnight at 4°C the precipitate was sedimented and the supernatant discarded. After twice washing the precipitate with 1 ml cold 10% TCA it was allowed to dry at room temperature and finally dissolved in hot (70°C) 1N NaOH. The total protein content of this solution was estimated by the method of LOWRY et al. (1951), using bovine serum albumin as a standard.

4. Counting of radioactive samples.

Radioactivity of cell and incubation medium proteins was determined by liquid scintillation counting using Bray's scintillation mixture (BRAY, 1960). 0.2 - 0.5 ml samples of the solubilised protein was added to 10 mls scintillation fluid. All samples were counted in duplicate for at least 10 min. In preliminary experiments the efficiency of counting was estimated using an internal H3 standard of known d.p.m. This was the same for all samples provided the total volume remained constant.

RESULTS

1. Cell isolation technique.

Preliminary experiments in which the technique of FLATLAND et al. (1969) was applied to the guinea pig and rat pancreas were unsuccessful. In both species the amount of force required to disrupt the tissue was too great to allow the recovery of intact cells.

Digestion of the guinea-pig pancreas with crude collagenase and pronase yielded suspensions of cells 90-95% of which could be identified as acinar cells on the basis of prominent secretory granules (PLATE 1). The major contaminants were red blood cells the proportion of which could be reduced by a more vigorous washing of the tissue after removal from the animal. In addition small cells were present, some of which were granulated. Presumably these were derived from ducts, blood vessels, connective tissue or the islets of Langerhans. Apart from washing the tissue prior to digestion no further attempt was made to obtain a purer preparation of acinar cells.

Isolated guinea-pig acinar cells were rounded measuring approximately 20 μ in diameter and maintained their *in situ* polarity of apical zymogen granules and basal nuclei (PLATE 1). They also demonstrated some degree of cytoplasmic blebbing and tended to reaggregate in suspension although acinar like structures were never reformed. On the basis of the Eosin Y dye exclusion test 95% of the cells were usually viable at the time of isolation and maintained both viability and morphological characteristics throughout all the test procedures described.

A similar digestion technique employing crude collagenase alone

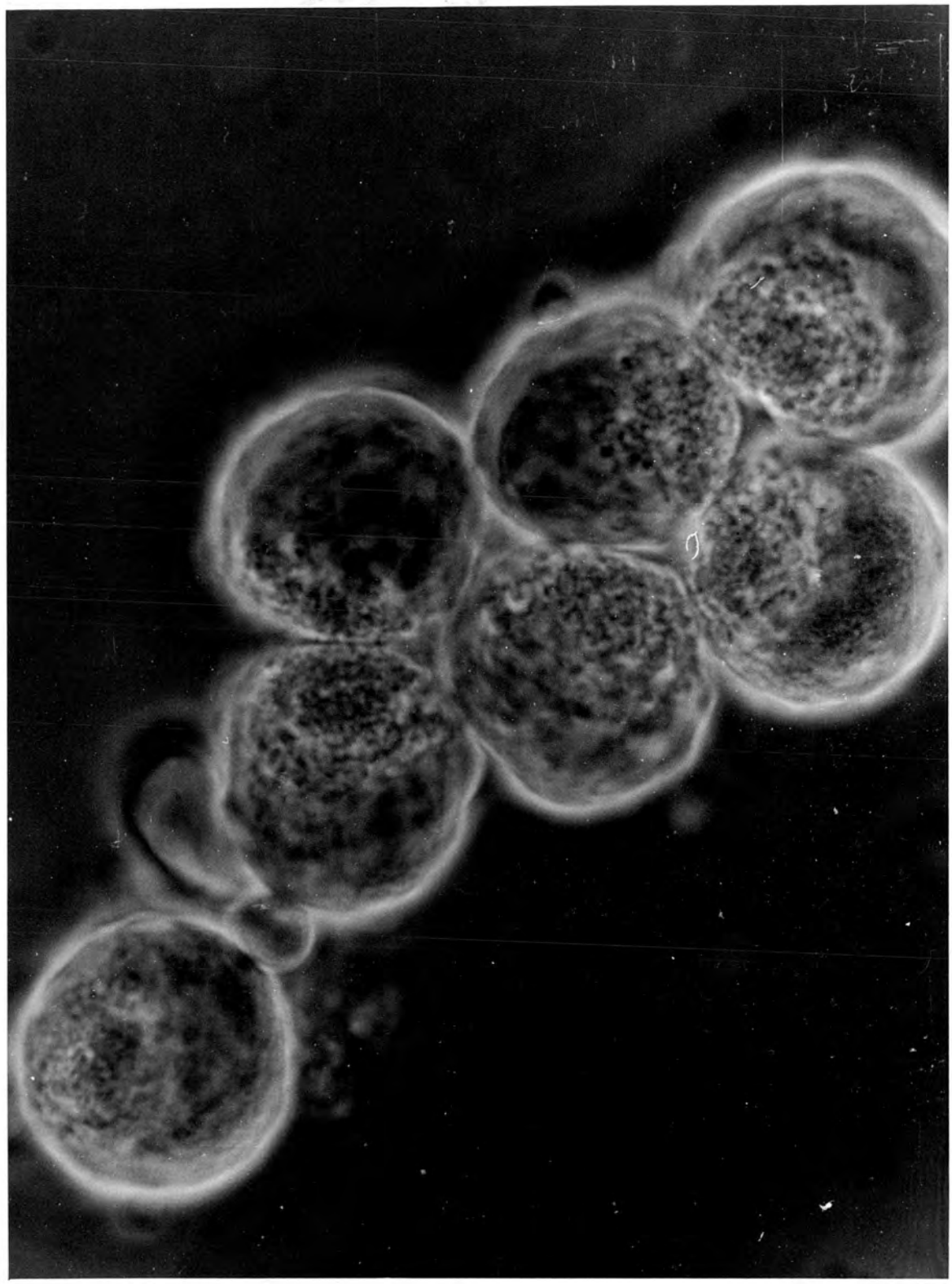

PLATE I

Acinar cells isolated from the guinea pig pancreas by collagenase and pronase digestion (Phase contrast x 2100).

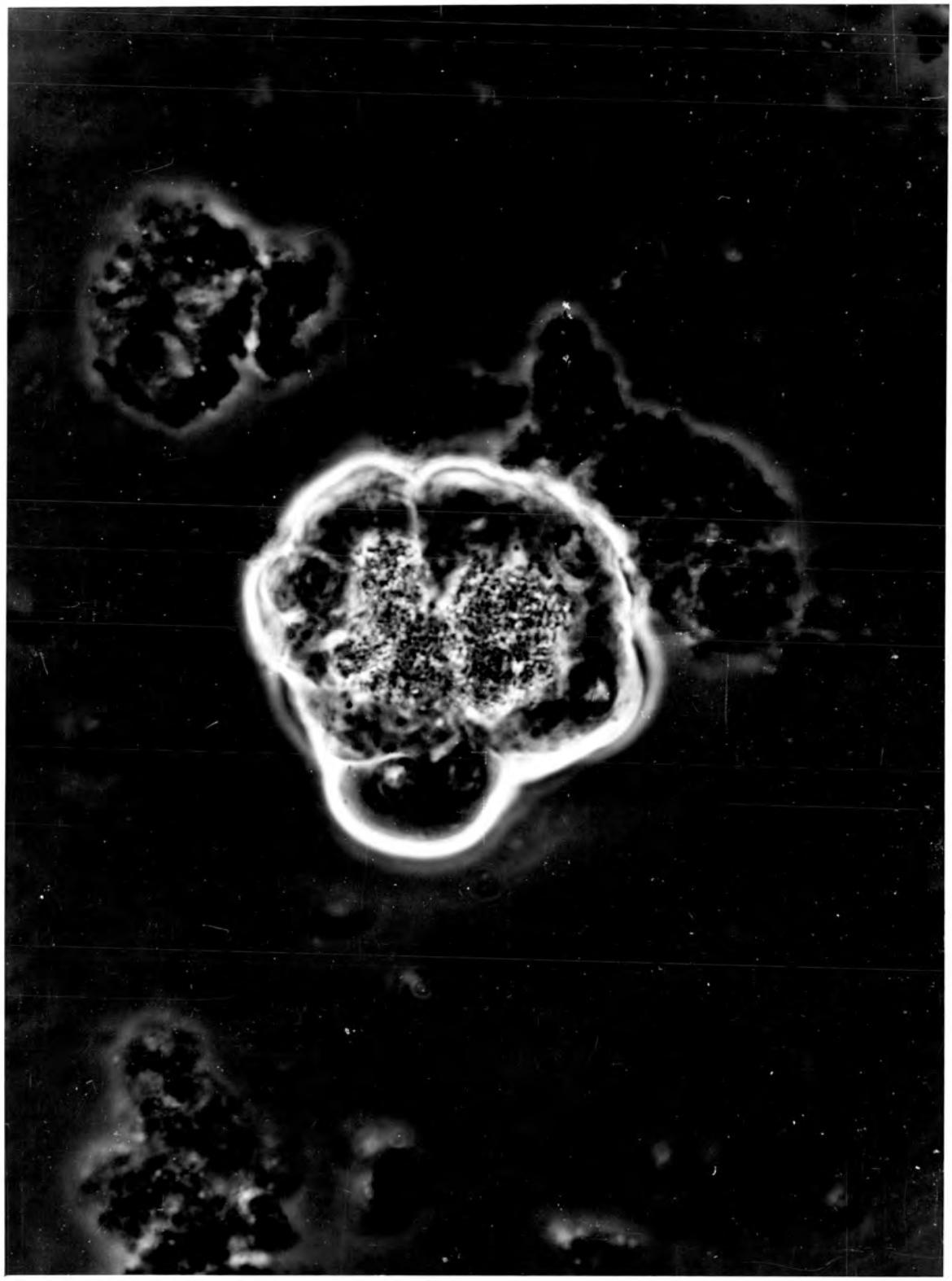

PLATE 2

An isolated acinus obtained by collagenase digestion of a rat pancreas. (Phase contrast x 1000)

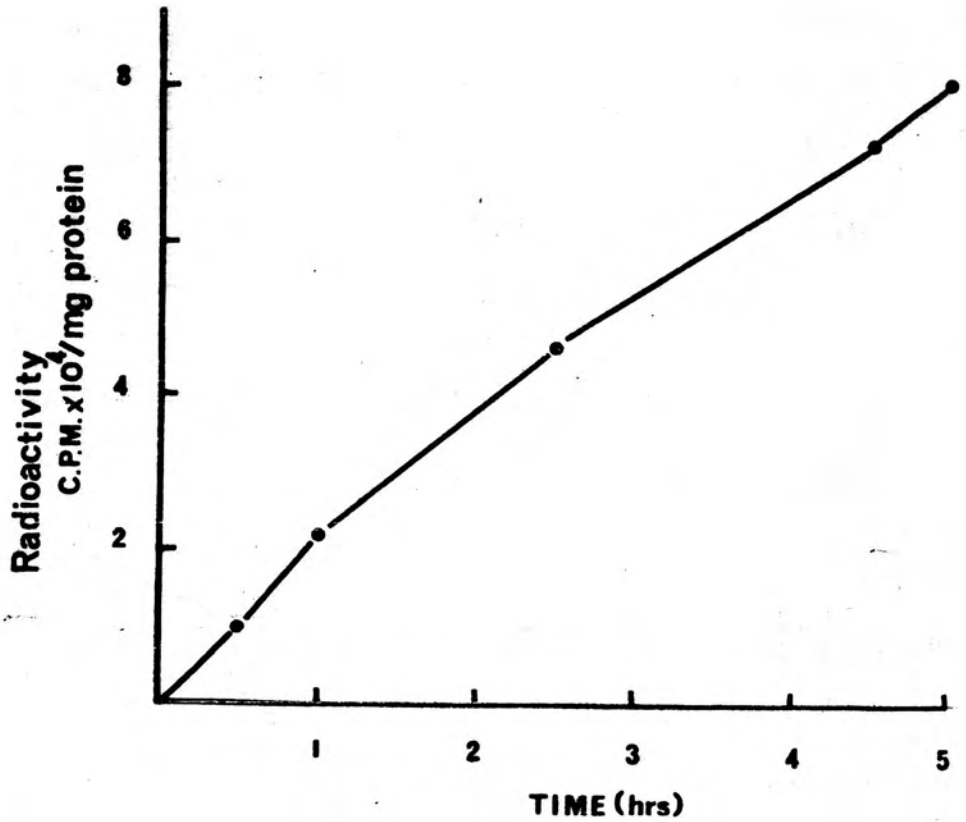

FIG. 30

The incorporation of L-Leucine-4-5-H3 into cellular proteins by isolated acinar cells obtained from the guinea pig pancreas. One experiment.

TABLE III. The secretion of de novo synthesised proteins by isolated acinar cells derived from the guinea pig pancreas. After pulse labelling with L-leucine-4-5-H3 the cells were incubated for 2 hrs in the presence or absence of CCK-Pz, acetylcholine or carbamylcholine at the concentrations indicated. The protein bound radioactivity present in the supernatant after 2 hrs is expressed as a percentage of the total protein bound radioactivity.

STIMULANT	Percent of total protein bound radioactivity secreted TEST	CONTROL	T-C	N
PZ-CCK 5 Units/ml	25.6	6.8	18.8	4
PZ-CCK 1 Unit/ml	13.3	9.5	3.8	1
ACETYCHOLINE (10^{-4}M)	8.5	6.4	2.1	2
ACETYLCHOLINE (10^{-4}M) + ESERINE (10^{-5}M)	5.8	3.5	2.3	1
CARBAMYLCHOLINE (10^{-4}M)	6.1	4.1	2.0	2

was effective for the isolation of single acini from the pancreas of PVG strain but not Wistar rats (PLATE 2). However the yields were considered too low for biochemical studies.

The yield of single acinar cells from the guinea pig (gland wet weight 0.8 - 0.9g) varied between 50 and 100 mg cells wet weight i.e. approximately 5-10% and was always greater than that obtained from rats. In addition cells isolated from rats exhibited a much greater tendency to clump together. Collagenase or pronase alone were both effective in isolating acinar cells from the guinea pig pancreas although the yields were much greater when the two enzymes were combined. Neither trypsin, pancreatin or hyaluronidase proved effective substitutes for pronase.

2. Incorporation of L-leucine-4-5-H3 into cell proteins.

Isolated guinea-pig acinar cells incorporated L-leucine-4-5-H3 into cellular proteins at an approximately linear rate for 5 hrs after isolation (Fig. 30). Longer incubation times were not tested.

3. Secretion of de novo synthesised protein in response to CCK-Pz, acetylcholine and carbamylcholine.

The results of these experiments are shown in Table III. Controls indicated that the basal secretion varied in different experiments with an average of 6% (10) of the total protein bound radioactivity appearing in the supernatant during the 2 hr incubation. Addition of crude CCK-Pz (5 C.H.R. units/ml) in 4 experiments increased the secretion of labelled protein by a mean of 18.8%. In one experiment a proportional response, 3.8%, was achieved with 1 C.H.R. unit CCK-Pz/ml. Acetylcholine (10^{-4}M) was much less effective as a

secretory stimulant. This was not due to the hydrolysis of acetylcholine by cholinesterase since neither the addition of eserine (10^{-5}M) nor the substitution of carbamylcholine which is resistant to cholinesterase (KOELLE,1970) increased the stimulation.

DISCUSSION

1. Cell isolation technique

Although there is considerable evidence to suggest that cells subjected to mechanical stress during isolation are damaged (see introduction) an attempt was made to obtain pancreatic acinar cells using the technique described by FLATLAND et al. (1969). The degree of mechanical force required to disrupt the pancreas was however inconsistent with the maintenance of cellular structure.

The conditions finally adopted for the enzymic separation of acinar cells were in part arrived at by empirical means and in part conditioned by previous experience with the isolated perfused cat pancreas. In these experiments it was shown that prolonged exposure of the gland to calcium free solutions containing EGTA resulted in abnormalities of enzyme secretion that were only partially reversible. Because of this the use of such a procedure was avoided in the cell isolation experiments. A perfusion technique was also considered, but discarded, since it was felt that this would add unnecessarily to the complexity of the procedure. Instead access of the digesting enzymes to the intercellular spaces was ensured by finely chopping the tissue. The unavoidable contamination with blood cells could be substantially reduced by washing the tissue fragments prior to incubation.

In addition to collagenase a proteolytic activity was necessary to obtain reasonable yields of acinar cells. Trypsin proved unsatisfactory since it resulted in the formation of mucilagenous strands which hampered resuspension of the isolated cells - an observation also made by MADDEN & BURK (1961). Pancreatin was

ineffective and hyaluronidase, although increasing the yield, tended to render the cells extremely fragile which resulted in the formation of large amounts of debris. Pronase proved the most effective. This may be due to the fact that the enzyme, isolated from Streptomyces griseus, possesses the broadest substrate specificity of any known protease (GWATKIN & THOMSON, 1964). Its effectiveness may have been enhanced since it combines proteolytic and mucolytic activity (BLUM, HIRSCHOWITZ, HELANDER & SACHS, 1971). Mild mechanical treatment was also necessary to effect dispersion of the pancreas and this was provided by shaking the tissue pieces during incubation and by pipetting the fragments which remained after digestion through a wide bore pasteur pipette.

The technique described by AMSTERDAM & JAMIESON (1972, 1974a) for the isolation of guinea pig acinar cells contains in addition to enzymic digestion and mechanical disruption a step involving chelation of divalent cations. By examination of the tissue at various stages in the isolation procedure the authors have been able to define the role played by each step in the liberation of isolated cells. (AMSTERDAM & JAMIESON, 1974a). Exocrine cells of the pancreas are joined laterally by junctional complexes comprising in an apical basal direction: tight junctions, zonulae adherentes and desmosomes together with a large gap junction on the lateral plasmalemma about two thirds of the distance from the apex to the base. Removal of divalent cations, by incubation in either calcium and magnesium free media or EDTA, cleaved the desmosomes and separated a proportion of the tight junctions and zonulae adherentes. Gap junctions were resistant to EDTA treatment and required, as did the tight junctions and zonulae adherentes, application of mechanical force for complete

separation. Of the enzymes, collagenase, chymotrypsin and hyaluronidase, employed by these workers only collagenase caused a fine structural alteration of the pancreas by dissolving basement membrane fibrils. It was suggested that chymotrypsin acted by removing protein bound groups from the cell membrane that might directly or indirectly through ligands such as calcium effect cell adhesion. Omission of hyaluronidase had no significant effect on the dissociation procedure.

It can thus be concluded that divalent cation removal and mechanical forces are major factors causing the separation of cell to cell contacts in the pancreas. In the present study chelating agents and calcium free media were not employed and mechanical forces were kept to a minimum, factors which may explain why the yields obtained (10% by wet weight) were lower than those reported by AMSTERDAM & JAMIESON (1972, 1974a) of 50-60% based on DNA recovery. Increasing the time for which the tissue was exposed to pronase and collagenase tended to increase the yield of cells but also reduced their viability as judged by eosin Y dye exclusion.

2. Morphology of isolated acinar cells.

Phase contrast microscopy of the cell suspensions indicated that 90-95% of the population consisted of acinar cells. Although the cells tended to aggregate somewhat acinar like structures were never reformed. The cells were rounded, but maintained their *in situ* polarity of apical zymogen granules and basal nuclei. This polarity also extends to the plasma membrane which retains its apical microvilli (AMSTERDAM & JAMIESON, 1974a). The only structural abnormality detected by phase contrast microscopy of freshly isolated

suspensions was the presence of cytoplasmic blebs, attached to the cells. Electron microscope (EM) studies on isolated hepatocytes (BERRY & FRIEND, 1969) and pancreatic acinar cells(AMSTERDAM & JAMIESON, 1974a) have identified these blebs as consisting of areas of plasma membrane attached to intact gap and tight junctions. Acinar cells also show some swelling of the golgi apparatus and an increased number of condensing vacuoles. BERRY & FRIEND (1969) have also reported that hepatocytes occasionally exhibit large vacuoles located in the region of the golgi complex. Since the same cells also lacked gap and tight junctions they have postulated that these vacuoles may represent an outward transport process for extracellular fluid absorbed when such junctions are broken by mechanical stress.

In the present study there was no apparent change in the phase contrast morphology of the cells during the course of experiments in which they were incubated for up to five hours. EM studies have indicated an additional increase in the number of condensing vacuoles and autophagic bodies over that observed immediately after isolation (AMSTERDAM & JAMIESON, 1974a).

3. Viability of isolated acinar cells.

During development of the dissociation technique an Eosin Y dye exclusion test was used as a rapid method for estimating the viability of cell preparations (HANKS & WALLACE, 1958). Although this test only measures the passive properties of the cell membrane which might persist after cell damage it is a reliable screening method and allows immediate assessment of the quality of the cells. Normally 95% of the isolated acinar cells in a given preparation excluded the dye. In addition isolated acinar cells incorporated

radioactive L-leucine into protein, secreted labelled protein in response to CCK-Pz and multiplied in culture (see below). Although these observations suggested that the cells were viable and retained some of their *in situ* characteristics they do not provide quantitative information on the degree of viability compared with the intact gland.

4. Incorporation of L-leucine into cellular protein.

In a series of elegant studies on the synthesis and intracellular transport of proteins JAMIESON & PALADE (1967a, b) have shown that radioactive leucine is incorporated into cellular proteins by the guinea pig pancreas. Isolated acinar cells performed the same function and the rate of incorporation remained linear for at least five hours. Since the experiments were not performed under sterile conditions it could be argued that this incorporation reflected bacterial contamination of the cell suspensions. Three observations weigh against this interpretation. Firstly a proportion of this labelled protein was discharged into the medium in response to a stimulant of enzyme secretion; secondly, isolated acinar cells incorporate L-leucine into protein in the presence of antibiotics; thirdly, autoradiographic studies have identified acinar cells as the site of incorporation (AMSTERDAM & JAMIESON 1974b). The data presented in Fig. 30 are not directly comparable with the rates of incorporation previously observed in the intact gland since these have been expressed on a DNA basis. However AMSTERDAM & JAMIESON (1974b) have shown that isolated acinar cells incorporate amino acids at rates higher than the intact tissue:an observation probably related to the limiting effect of diffusion barriers in slice systems.

The same workers have also reported that all the acinar cells of a given population were equally active in incorporating amino acids into proteins and that the synthetic route followed (rough endoplasmic reticulum - golgi peripheral region - condensing vacuoles - zymogen granules) was the same as in the intact tissue. The kinetics of this intracellular transport were also similar to intact glands except for a defect which existed in the terminal conversion of condensing vacuoles to zymogen granules. This defect resulted in the whole cycle being delayed by about one hour.

5. Secretion of labelled proteins.

The ability of isolated acinar cells to secrete labelled exportable protein in response to secretagogues was tested. Suspensions of cells were pulse labelled with H3-L-leucine and after allowing sufficient time for incorporation of the label into protein and for transport to the zymogen granules exposed to secretagogues. Estimation of the protein bound radioactivity in the medium provided a simple sensitive assay of secretory potential which required in addition simultaneous function of the synthetic apparatus.

The results indicated that the cells responded to CCK-Pz but that acetylcholine and carbamylcholine were much less effective. AMSTERDAM & JAMIESON (1974b), using a similar technique, observed that the amount of labelled protein secreted depended on the procedure used for chase incubation and on the time interval between the pulse and the addition of secretagogues. If added immediately after the pulse, 10^{-8}M caerulin stimulated secretion of 25% of the total protein bound radioactivity (TPBR) in two hours. Only 12% of

the TPBR was secreted when the hormone was added after one hour of chase incubation. Under similar conditions controls secreted about 3% of the TPBR. Since this dose of caerulin gave a maximal secretory response in their experiments it would appear that the preparation described here is rather more sensitive to peptide hormones. In four experiments 25.6% of the TPBR was secreted during a two hour exposure to CCK-Pz following a 60 min chase incubation. In the same series of experiments controls secreted 7% of the TPBR. This increased responsiveness (approx. x 2) may be related to the fact that these experiments were performed in a complex culture medium containing calf serum whereas AMSTERDAM & JAMIESON (1972, 1974a,b) employed the simpler Krebs bicarbonate ringer supplemented with amino acids and bovine serum albumin. Furthermore these cells were not exposed to divalent cation removal and chelating agents:a procedure which causesirreversible inhibition of enzyme secretion from the perfused cat pancreas (see part I of this thesis).

In contrast to the effects of CCK-Pz the isolated acinar cells were virtually unresponsive to cholinergic drugs. Conversely AMSTERDAM & JAMIESON (1972, 1974b) have reported that carbamylcholine (10^{-4}M) elicits a secretion similar in magnitude to that obtained with a maximal dose of caerulin (10^{-8}M). The concentrations of secretagogues used in all these studies are very high, about ten times higher than those required for optimal release from pancreatic slices (JAMIESON & PALADE, 1971 a,b) and lobules (SCHEELE & PALADE, 1975). In addition the net release is about 50% less than that described for the intact tissue. This suggests a malfunction in the secretory process. Since the secretion of preformed amylase parallels the secretion of radio-labelled protein (AMSTERDAM & JAMIESON, 1974b)

It can be concluded that a defect may exist at the level of the hormone receptors, which are presumably protein complexes located at the cell surface (CUATRECASAS, 1973). It is well known that hormone receptors in other cell systems are susceptible to proteolytic hydrolysis (CUATRECASAS, 1972; KONO, 1969) and some loss of function may be an unavoidable consequence of the procedures employed to isolate the acinar cells.

The observation that the cells used in this study were responsive to CCK-Pz but not to cholinergic agents poses a number of interesting questions. Provided the assumption is made that the secretory pathway stimulated by these agents is the same it can be concluded that a rather specific inactivation of the acetylcholine receptor has occurred. The origin of this inactivation presumably lies in the differences between the cell isolation technique described in this thesis and that developed by AMSTERDAM & JAMIESON,(1972, 1974a). The latter authors employed collagenase, hyaluronidase and chymotrypsin digestion, divalent cation removal and mechanical forces. The present technique employs collagenase and pronase digestion and mechanical forces. It seems unlikely that omission of divalent cation chelation is the cause, if anything the reverse would be expected. A possible explanation is that the pronase contained a proteolytic activity deleterious to the muscarinic acetylcholine receptor complex but which had less effect on the CCK-Pz receptor complex. The binding of acetylcholine to proposed cholinergic receptors isolated from a number of tissues is affected by proteolytic and in some cases lipolytic enzymes (O'BRIEN, ELDEFRAWI & ELDEFRAWI 1972).

PART III

PANCREATIC CELLS IN CULTURE

INTRODUCTION

The observation that mammalian pancreas was capable of survival and differentiation in organ culture was made by CHEN (1954). Since then many other workers have reported the *in vitro* survival or development of pancreas explants from a variety of species (see MURRELL, 1966). Generally the early studies dealt only with the morphology of pancreatic acinar and islet tissue but more recently foetal organ cultures have been used to study acinar cell differentiation and its regulation (WESSELLS, 1964; PARSA, 1973). These experiments have shown that foetal organ culture accurately reflects differentiation of the rat pancreas *in vivo* both structurally and in terms of accumulation of the digestive enzymes lipase, amylase and chymotrypsin (PARSA, MARSH & FITZGERALD, 1969).

The successful growth of monolayer cultures from dispersed pancreatic cells has been reported from three laboratories. (HILWIG, SCHUSTER, HEPTNER & VON WASIELEWSKI, 1968; MACCHI & BLAUSTEIN, 1969; HILWIG & SCHUSTER, 1970; HILWIG & VRBANEC, 1970; LAMBERT, BLONDEL, KANAZAWA, ORCI & RENOLD, 1972; ORCI et al. 1973). These workers share a common interest in the culture of endocrine elements of the pancreas. The A and B cells of the islets of Langerhans make up a small volume of the intact gland and the establishment of cultures rich in these cells provides many advantages for the study of insulin and glucagon secretion. Their results indicate that the B and A endocrine cells survive and multiply *in vitro* retaining their morphological characteristics and the ability to synthesize and secrete insulin and glucagon both spontaneously and in response to a number of physiological stimulants (MARLISS, WOLLHEIM, BLONDEL, ORCI,

LAMBERT, STAUFFACHER, LIKE & RENOLD, 1973).

The exocrine acinar cells however appear to adapt less well to *in vitro* conditions. They undergo necrosis and progressive dedifferentiation during the first few days in culture becoming unspecialised epithelial cells (ORCI et al. 1973). One aim of the present investigation was to discover how far this dedifferentiation had proceeded by examining a property of the cells, the electrophysiological response to stimulation, which is independent of morphological and enzyme synthesizing characteristics. As a first step the isolated preparation of acinar cells described previously had to be grown in culture and this forms the subject of the following section.

METHODS

A. Cell Culture Technique

1. The Laboratory

Except for some preliminary experiments all the tissue culture work was carried out in an aseptic room. This consisted of a small laboratory which had been thoroughly cleaned and repainted. To minimise the ingress of airborne contaminants the windows remained closed and ventilation was provided by an extractor fan. Bench tops were covered with polythene sheeting. The bench area being used was washed with 70% ethanol prior to starting the experiment. A tissue culture hood provided a covered area under which the cultures were manipulated. Given these conditions and careful sterile technique the loss of cultures through bacterial and fungal infections was low enough to permit the experiments to be performed without addition of antibiotics to the culture media. Any cultures that became infected were immediately removed from the laboratory.

2. Preparation of Apparatus.

(a) Washing of glassware and plastic:- All glassware and reusable plastic equipment was cleaned by soaking in Decon 75 (1 in 200 V/V solution) for a minimum period of 12 hours. The advantage of this detergent is that it is highly efficient and relatively easily rinsed off. After treatment the articles were thoroughly rinsed in tap water and then at least 3 times in distilled water.

(b) Sterilization of apparatus and solutions:- All glassware was sterilized by dry heat. The article was wrapped in aluminium

foil and heated to 160°C for 90 mins.

Plastic and rubber articles were sterilized by washing in 70% ethanol sealed while still wet in a paper bag and dried in a 40°C oven.

Millipore filters (cellulose acetate HA 0.45 μ) were autoclaved preassembled in a 'Swinnex' syringe holder.

Pronase and collagenase solutions used for acinar cell isolation were sterilized by millipore filtration.

3. Culture media and sera.

Sterile culture media and sera were purchased from Biocult Laboratories Ltd. In preliminary experiments a number of different media were tested for their ability to support cell growth viz: Eagle's MEM, Ham's F12, NCTC 109, McCoy's 5A. All media were supplemented with 20% calf serum.

4. Preparation of hydrated collagen lattices.

In experiments where the electrophysiological characteristics of the cultured cells were examined they were grown on hydrated collagen lattices prepared by the method of ELSDALE & BARD (1972). In addition to providing an excellent and essentially transparent substratum for cellular growth the collagen prevents damage to micropipettes caused by striking the bottom of the culture dish. Their preparation briefly described below is in two phases, solubilization of rat tail collagen followed by precipitation of the hydrated lattices.
(a) Preparation of collagen solutions:- The aim is to prepare clean stable collagen solutions utilizing the principle that these solutions are stable at low ionic strength and low pH.

(i) Rat tails were first sterilized by immersion in 95% alcohol for 15 mins.

(ii) The tendons were dissected out and extracted in 0.5M acetic acid at $4^{o}C$ for 2 days with stirring (100-200 mls acetic acid per tail).

(iii) This extract was filtered through two layers of butter muslin.

(iv) 100-300 mls of the filtered extract was dialysed for 24 hrs against two 4 l batches of one-tenth strength Eagle's MEM without bicarbonate. The second batch of dialysate was adjusted to pH 4 with HCl.

(v) This dialysed solution was centrifuged at 10 x $10^{3}g$ for 24 hrs. The almost clear supernatant so obtained was always sterile and kept indefinitely at $4^{o}C$.

(b) Precipitation of hydrated collagen lattices:- The collagen was caused to precipitate and aggregate into native bundles by raising the ionic strength and pH to physiological levels. To avoid a useless flocculation it was necessary to adjust the pH and ionic strength almost simultaneously.

(i) A sample of the collagen solution was cooled on ice.

(ii) Three syringes were prepared containing appropriate quantities of calf serum and 10 x Eagle's MEM to reconstitute the standard medium and a predetermined volume of sterile 0.1N NaOH to bring the pH of the mixture to 7.6.

(iii) Working quickly the three ingredients were added to the collagen solution and the mixture dispensed into 20 mm Falcon plastic tissue culture dishes. Enough of the mixture was added so as to just cover the bottom of the dish. Care was taken to prevent the

inclusion of air bubbles.

(iv) The dishes were left undisturbed for about 10 mins while the mixture set. Freshly prepared collagen solutions set quickly, stored solutions set conveniently, more slowly.

5. Preparation of primary cell cultures.

Isolated acinar cells were prepared by collagenase and pronase digestion of the guinea pig pancreas, as described except that sterile technique was adhered to throughout. After isolation and eosin Y viability testing the cells were counted using a haemocytometer and diluted with culture medium to an inoculation density of 10^5 - 10^6 cells/ml. Falcon 20 mm dishes were innoculated with approx. 2 mls of the cell suspension. Normally 10-15 such cultures were established per experiment either in plain or collagen coated dishes.

6. Maintenance of primary cultures.

The primary cultures were sealed in a plastic box containing a humidified atmosphere of 5% CO_2/95% Air and incubated at 37°C. The pH of the cultures could be monitored visually since the medium contained phenol red indicator. Daily inspections were carried out to identify and remove any infected cultures. Every three days the cultures were fed by complete replacement of the growth medium. Primary cultures 3-5 days old were used in all subsequent experiments.

7. Preparation of cultures containing only epithelial cells.

Primary cultures prepared by the above techniques contained

cells of two morphologically distinct types, epithelial and fibroblastic. The epithelial cells tended to grow slowly in culture and were eventually overrun by fibroblasts. An attempt was made to obtain cultures consisting solely of epithelial cells. Two approaches were employed, the establishment of cultures from single acinar cells (cloning) and the subculture of existing epithelial colonies from 3 day primary cultures.

(a) Cloning cells by the isolation technique.

The method described by PAUL (1970) was used. A Falcon culture dish was filled with liquid paraffin saturated with culture medium (McCoy's 5A + 20% calf serum). This was prepared by shaking a mixture of paraffinum liquidum B.P. and culture medium (10:1 V/V) in a sterile bottle. This mixture was then incubated at 37°C until the paraffin had cleared (48 hrs). Four small drops of the culture medium were then placed around the dish. The drops fall through the paraffin and spread out as discrete areas on the bottom of the dish. Using a micropipette, prepared from a pasteur pipette and working under a microscope freshly isolated guinea pig acinar cells were individually transferred to the drops of culture medium. Each drop received between 1 and 10 cells. The cultures were then incubated in an humidified air 95% / CO_2 5% atmosphere at 37°C and examined after 2 days. On every subsequent day the cultures were fed by replacing a proportion of the drop with fresh culture medium.

(b) Subculture of epithelial cell colonies.

Guinea pig acinar cell cultures were prepared in Falcon dishes the bottoms of which were covered with small pieces of broken cover-slip glass. After 72 hrs incubation the cultures were inspected in a Prior inverted microscope and using sterile technique pieces of

glass containing adhered epithelial cell colonies removed to drop cultures under liquid paraffin (one colony per drop). Incubation and maintenance of the cultures was as described for cloning.

8. Microscopy of cultures.

Cultures were routinely inspected using a Prior inverted microscope fitted with a long working distance condenser. After washing the cultures phase contrast micrographs were taken with a Wild M40 inverted microscope.

9. Presence of amylase in cultures.

5 day primary cultures were washed three times with isotonic NaCl at 37°C. The cells from several cultures were harvested by scraping them from the bottom of the dish and homogenised in a small volume of 1.5% NaCl. The homogenate was assayed for amylase by the Nørby method (LAGERLOF 1942).

RESULTS

1. Growth of pancreatic cells in culture.

Suspensions of isolated cells obtained from the guinea-pig pancreas by collagenase and pronase digestion were successfully cultured in a growth medium consisting of McCoy's 5A + 20% calf serum. NCTC 109 and Ham's F12 also supported growth but were less effective than McCoy's 5A. No growth was observed when the simpler Eagle's MEM was employed.

The isolated cells adhered to the Falcon dishes and two dimensional colonies were identifiable after a lag phase of 24-48 hrs. Primary cultures innoculated at a density of 5×10^5 cells/ml formed confluent monolayers by the 7th day. There appeared to be no differences in the morphology of cells cultured on plastic or collagen substrata.

2. Morphology of the cultured cells.

Phase contrast microscopy identified cells of two morphologically distinct types.

(a) Epithelial cells: 5 days post innoculation these cells were present as discrete colonies. They took the form of sheets of cells possessing a 'crazy paving' like arrangement. Their cytoplasm had a regular outline with few processes and was often finely granular (PLATES 3 & 4). One or more dense particles (presumably nucleoli) were contained within the nucleus. Occasionally large solitary epitheloid cells were observed (PLATE 5).

(b) Fibroblastic cells: Numerically these were always the largest population of cells in the cultures. Characteristically they

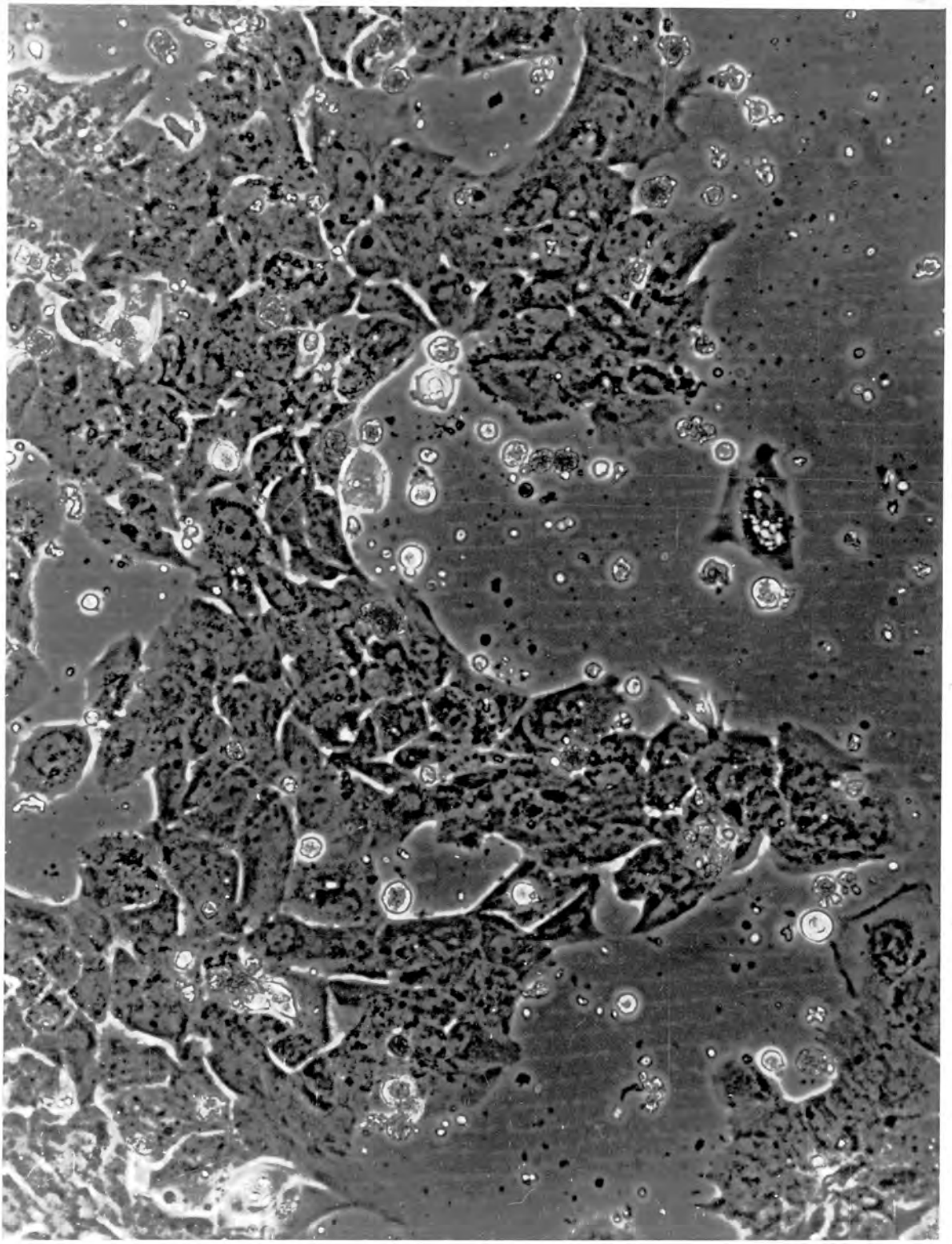

PLATE 3

An epithelial cell colony in a 5 day primary culture of pancreatic cells derived from the guinea pig. The cells were cultured on a plain 'Falcon' dish in a medium consisting of McCoy's 5A + 20% calf serum. (Phase contrast x 285).

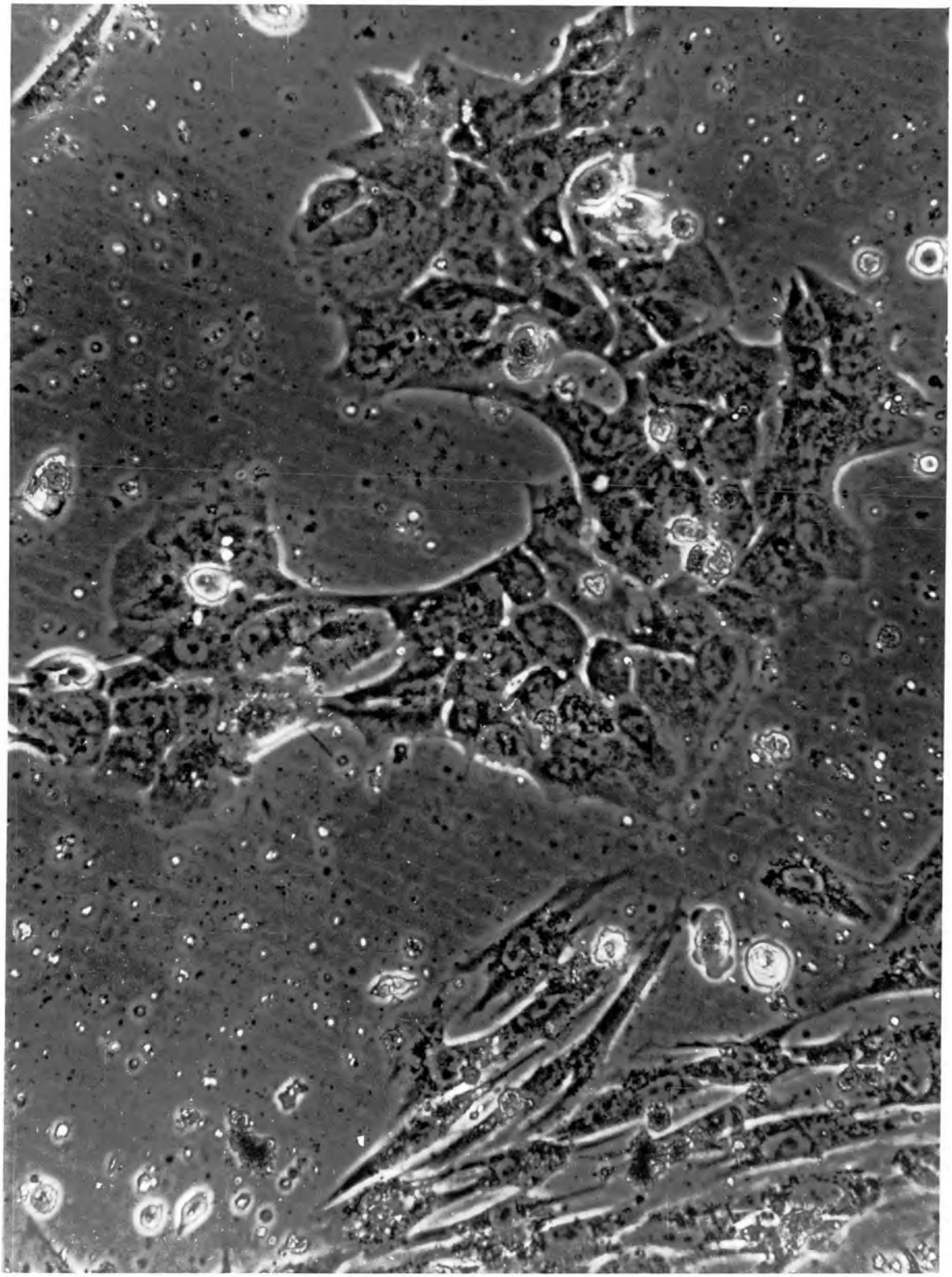

PLATE 4

An epithelial cell colony in a 5 day primary culture of pancreatic cells derived from the guinea pig. The lower right of the field shows the periphery of a fibroblast colony. The cells were cultured on a plain 'Falcon' dish in a medium consisting of McCoy's 5A + 20% calf serum. (Phase contrast x 285)

PLATE 5

Solitary epithelial cells and fibroblasts in a 5 day primary culture of guinea pig pancreatic cells. The cells were cultured on a plain 'Falcon' dish in a medium consisting of McCoy's 5A + 20% calf serum. (Phase contrast x 285)

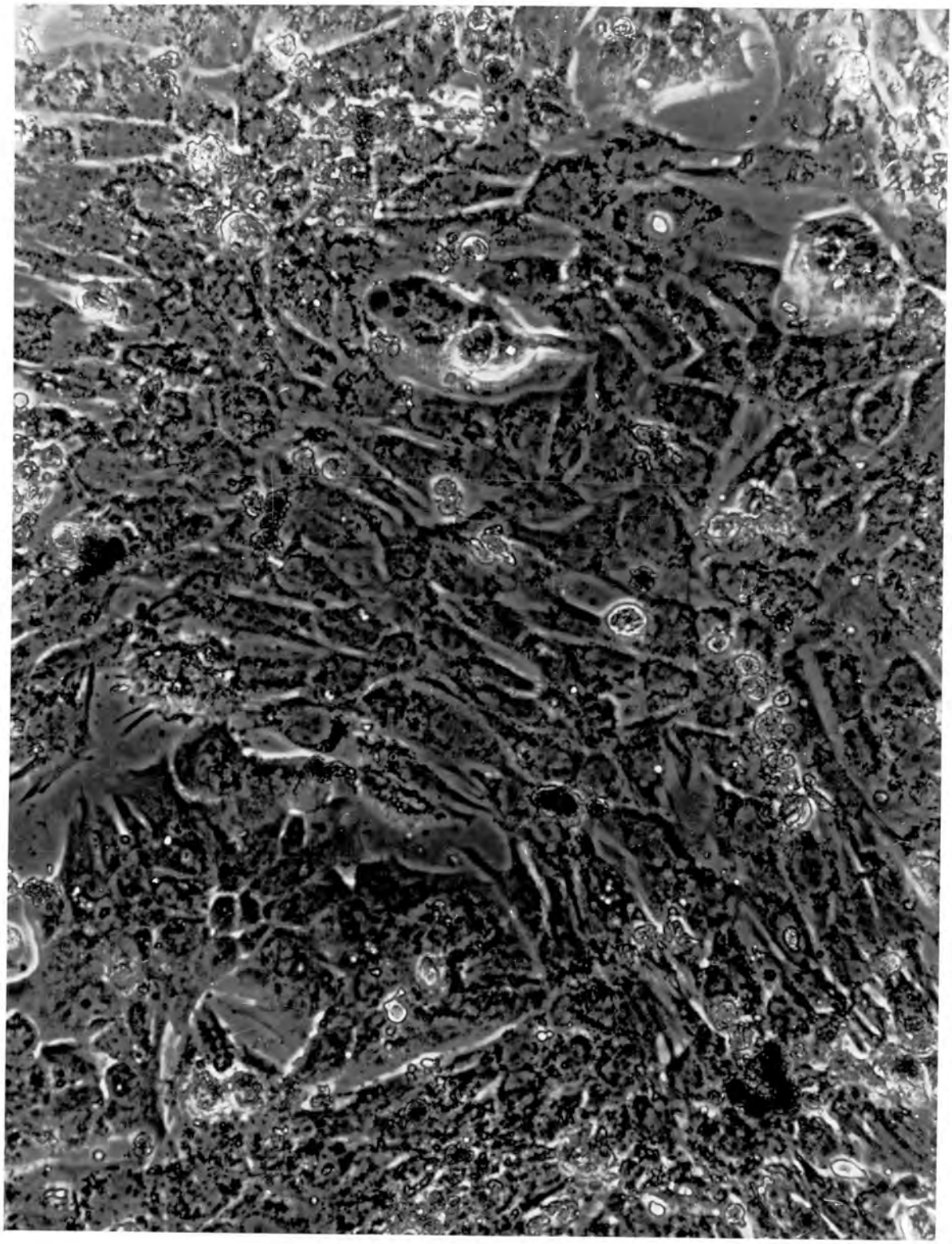

PLATE 6

A confluent area in a 5 day primary culture of guinea-pig pancreatic cells. The field contains an epithelial cell colony surrounded by sheets of fibroblasts. The cells were cultured on a plain 'Falcon' dish in a medium consisting of McCoy's 5A + 20% calf serum. (Phase contrast x 285)

were long thin cells with numerous filamentous processes. (PLATES 4, 5 & 6). The cytoplasm contained coarse granules which were usually confined to the central region of the cell surrounding the nucleus. The large nucleus contained one or more nucleoli like bodies. These cells possessed the highest rate of growth and confluent areas showed islands of epithelial cells surrounded by sheets of fibroblasts (PLATE 6) eventually the fibroblasts completely overgrew the epithelial cells.

3. Cultures containing only epithelial cells.

Neither cloning nor the subculture of epithelial cell colonies was successful in producing cultures containing purely epithelial cells. Colonies were never established from single cells suggesting that the plating efficiency was extremely low. It was technically difficult to obtain subcultures consisting solely of epithelial cells and these appeared to cease growing when isolated.

4. Amylase in cultures.

Amylase was not detected in 5 day old cultures.

DISCUSSION

1. Growth of pancreatic cells in vitro

The results show that suspensions of adult guinea-pig pancreatic cells survived and multiplied in culture. This is in agreement with a number of previous reports using cell suspensions derived from, in addition to adult guinea-pig, foetal rat and man, neonatal rat and neonatal hamster pancreas (HILWIG et al., 1968; LAMBERT et al., 1972; MACCHI & BLAUSTEIN, 1969). The cells grew best in a more defined culture medium such as McCoy's 5A and no growth was observed in the simpler Eagle's MEM. It proved impossible to obtain cultures consisting of epithelial cells alone and there were always large numbers of rapidly growing fibroblasts present in the cultures. LAMBERT et al. (1972) have reported that it is possible to obtain cultures enriched in epithelial cells by taking advantage of the fact that fibroblasts adhere to the culture dish rather more rapidly than the epithelial population. Decantation of the primary cultures 14 hours after inoculation eliminated 80% of the initial fibroblast population but also resulted in a concomitant loss of 40-50% of the epitheloid cells.

2. Morphological characteristics.

On the basis of their morphological characteristics WILLMER (1960) has defined six cell types occurring in culture. These are epithelial, fibroblasts, amoebocytes, nerve cells, neuroglia and lymphocytes. Cells of epithelial morphology are generally considered to arise from the covering layers of a wide variety of ectodermal, endodermal and mesodermal sources whereas fibroblasts are of mesen-

chymal origin i.e. connective tissue. From this classification it could be concluded that the epithelial cell colonies present in cultures of pancreatic cells are likely to contain descendants of the acinar cells whereas the fibroblasts represent contaminants derived from mesenchyme. In addition the epithelial population would be expected to contain endocrine cells derived from the islets of Langerhans, together with exocrine duct cells. The large solitary epithelial cells occasionally observed in the cultures may be classified as amoebocytes and as such were probably derived from blood monocytes and tissue macrophages.

Although the epithelial cells were often observed to contain granules in their cytoplasm there appeared to be no observable polarity in their relationship to the nucleus as is seen for acinar cells *in situ* and in isolation. In addition the digestive enzyme amylase was not detectable in 5 day old cultures. These observations suggest that the acinar cells had dedifferentiated in the process loosing the ability to synthesise and store digestive enzymes.

This general conclusion is in agreement with that previously arrived at by ORCI et al. (1973) working with neonatal rat pancreas. Using electron microscopy to follow the fate of the acinar cells these authors have detected a gradual loss of morphological features characterised by a reduction in size and number of R.E.R. cisternae, loss of zymogen granules, and an increase in the amount of filamentous material in the cytoplasm, together with abundant microtubules and vesicles. After 60 hours in culture these changes had proceeded to such a degree that it was only rarely possible to recognise cells which possessed characteristic exocrine features. The dedifferentiated descendents were however present with 'great frequency' (ORCI et al.

1973). The endocrine cells however survived better in culture. After 5.5 days up to 47% of the epithelial cell colonies (in epithelial cell rich cultures) contained aldehyde-thionin positive cells suggesting that they were derived from B cells. The colonies varied in size from less than 20 to more than 100 cells and at least 5 per cluster were aldehyde-thionin positive. The number of clusters with aldehyde-thionin positive cells was quite variable among different experiments and no indication is given as to the average per cent of B cells per epithelial colony. A and D cells together with serotonin and gastrin producing cells were also identifiable, but in lower numbers than the B cells.

It thus seems probable that most of the epithelial cell clusters consisted of acinar cell descendents and that some of these colonies (up to 47%) contained B cells. This seems a reasonable conclusion when it is remembered that the pancreas consists of about 1% insular tissue and that the isolated cell suspensions consist of approximately 90% acinar cells. The reason for this dedifferentiation remains unknown but may represent an inadequacy of the culture medium. Whether this process could be reversed remains a point for speculation.

PART IV

ELECTROPHYSIOLOGICAL STUDIES ON PANCREATIC CELLS *in vitro*

INTRODUCTION

In recent years the electrophysiology of cells in culture has received considerable attention (Table IV). This combination of techniques has usually been chosen where technical difficulties impair recording from the cells *in situ*, where it is required to study developmental and hybridisation processes or specialised cell lines from an electrophysiological standpoint. Such an approach offers many advantages. Cultures can often be obtained containing only one cell type; populations of cells can be synchronised in the same phase of the mitotic cycle; the cell from which electrophysiological recordings are made can be identified visually and the ionic environment around the cells is uniform and easily manipulated. An additional advantage is that cells isolated from their neighbours in culture can be studied without the complication of cell to cell electrical connections (via tight junctions) which are characteristic of epithelia *in situ* (LOEWENSTEIN *et al.*, 1965). Only two reports exist in the literature describing the use of these techniques for the study of a secretory cell. DOUGLAS *et al.* (1967) and DOUGLAS & KANNO (1967) have demonstrated that chromaffin cells isolated from the adrenal medulla of gerbils and maintained in short term feeder cultures depolarize in response to various secretory stimulants.

The present investigation was prompted by the fact that morphological and biochemical data indicated that acinar cells de-differentiate when maintained in culture. It seemed relevant to investigate how far this process had proceeded by further comparison of the cells *in situ* and their descendants in culture. Since acinar cells *in situ* are depolarized by the action of CCK-Pz and acetyl-

choline (DEAN & MATTHEWS, 1972) it was decided to undertake an electrophysiological study of the cultured cells. The results show that the resting membrane potentials of the cells in culture are much lower than those recorded from the intact gland. However carbamylcholine and CCK-Pz caused reversible depolarisation of the cells whereas secretin had no effect. The conclusion from these preliminary results is that the cells retain at least one of their *in situ* characteristics and that with refinements of the culture technique it might eventually prove possible to maintain specialised acinar cells *in vitro*.

TABLE IV

CELL TYPE	RESTING E_m mV	TEMPERATURE ^oC	REFERENCE
HELA	-50	-	OKADA, Y. et al. Biochim. Biophys. Acta. 291, 116-126. (1973)
"	-15.5	37	BORLE, A.B. et al. Cancer. Res. 28, 2401-2405.(1968)
	-11.9	27	
	- 9.2	17	
L cells (mouse fibroblasts)	-16	36-38	NELSON, P.G. et al. J. Gen. Physiol. 60, 58-71. (1972)
" "	-15	20	LAMB, J.F. et al. J. Physiol. 213, 683-689. (1971)
Human Fibroblasts	-70 to -75	35	SWIFT, M.R. et al. J. Cell. Physiol. 71, 61-64.(1968)
Chick Embryonic fibroblasts (interphase cells)	-5	30	O'LAGUE, P. et al. Science. 170, 464-466. (1970)
Ehrlich mouse ascites tumour cells	-11.2	22-26	ANULL, F. J. Cell. Physiol. 69, 21-32. (1967)

CELL TYPE	RESTING E_m mV	TEMPERATURE ^oC	REFERENCE
Ehrlich mouse ascites tumour cells	-11.1	-	HEMPLING H.G. J. Cell & Comp. Physiol. 60, 181-198. (1962)
Neuroglia	-31		
Fibroblasts	-27.4		
Mesenchymal cells	-27.4	37	WARDELL W.M. Proc. Roy. Soc. B 165, 326-360. (1966)
Macrophages	- 9.3		
Nerve cells	-70		
Hela	-27.9		
Neurones	-50	26	HILD W. et al. J. Neurophys. 25, 277-304. (1962)
Neuroglia	-50 to -70		
Spinal ganglion cells	-55 to	37	CRAIN S.M. J. Comp. Neurol. 104, 285-329. (1956)
Glial cells	-5 to -10 or -40 to -45	37	VERNADAKIS A. et al. Brain. Res. 57, 223-228.(1973)

CELL TYPE	RESTING E_m mV	TEMPERATURE ^oC	REFERENCE
Human astrocytes	- 7.7	26	TRACHTENBERG, M.C. et al. Brain Res. 38, 279-298.(1972)
Guinea pig astrocytes	-28.2		
Myocardial muscle cells	-41	37	CRILL, W.E. et al. Am. J. Physiol. 197, 733-735.(1959)
Chick embryo heart cells	-56	36-37	DEHAAN, R.L. et al. J. Gen. Physiol. 52, 643-665.(1968)
" "	-59	35	LEHMKUHL, D. et al. Am. J. Physiol. 205, 1213-1220.(1963)
" "	-43.3	21	FANGE, R. et al. Acta. Physiol. Scand. 38, 173-183.(1956)
Chick myotubules	-5 to -80 dependent on size and maturity. Average for mature tubules -59.1	37	FISCHBACH, G.D. et al. J. Cell. Physiol. 78, 289-300.(1971)
Human meningioma	-20 to -10	30	PRIETO, A. et al. Science. 157, 1185-1187. (1967)

CELL TYPE	RESTING E_m mV	TEMPERATURE	REFERENCE
Mouse neuroblastoma C1300. Clone N-18	Growth phase log -14 late log: -35 Stationary: -40	35	PEACOCK, J. et al. Exptl. Cell. Res. 73, 367-377. (1972)
Neuroblastoma C1300	-22	35	NELSON, P. et al. Proc. Nat. Acad. Sci. U.S.A. 64, 1004-1010, (1969)
Neuroblastoma (N)	-36		
Fibroblasts (L)	-21	35	PEACOCK, J.H. et al. Exptl. Cell. Res. 79, 199-212. (1973)
N & L Hybrids	-27 to -32		
Neuroblastoma (N)	-24		
L1 cells	-47		
L2 cells	-47	35	MINNA J. et al. Proc. Nat. Acad. Sci. U.S.A. 68, 234-239. (1971)
N x L1 Hybrids	-27		
N x L2 "	-34		
L1 x L2 "	-42		

CELL TYPE	RESTING E_m mV	TEMPERATURE	REFERENCE
Neuroblastoma & L cell Hybrids			
N4TG1 & B82			
NL1F	-25		
NL8B	-30		
NL13C	-19		
NL14J	-21		
NL7AC	-26	35	KINNA, J. et al. Nature New. Biol. 235, 225-231. (1972)
N18TG2 & B82			
NL308	-25		
NL309	-21		
NL304	-25		
NL305	-26		
Adrenal Medullary Cells	-29.3	37	DOUGLAS W.W. et al. J. Physiol. 188, 107-120.(1967)
Ovarian tumour cells	-25 interphase - 6 mitotic phase	-	REDMAN, K. et al. Acta. Biol. Med. Ger. 28, 853-856. (1972)

CELL TYPE	RESTING E_m mV	TEMPERATURE	REFERENCE
Hamster lung cells	-26.8	37	SACHS, H.G. et al. Exptl. Cell. Res. 83, 362-366.(1974)
Baby Hamster kidney. (B.H.K. cells)	-50 to -55	37	SACHS, H.G. et al. J. Cell. Physiol. 80 347-358. (1972)

METHODS

1. Preparation of glass microelectrodes.

After cutting into 4-inch lengths, Jecons glass tubing (Type H15/10) was boiled for 15 mins in 50% HNO_3, rinsed with tap water and finally boiled twice in distilled water. Electrodes were pulled using the Palmer vertical microelectrode puller (Type 418/8115). Immediately after pulling, the tips of the microelectrodes were immersed in 3M KCl and allowed to fill by capillarity (CALDWELL & DOWNING, 1955). Distilled water was pipetted into the electrode shaft, a small bubble being left between this and the KCl in the tip. Generally the lower part of the column of distilled water ended at the point where the electrode began to taper and the column extended two thirds of the way up the electrode shaft.

The electrodes were then stored for at least 48 hrs their shafts being pressed into plasticine round the rim of a beaker such that their tips were immersed in 3MKCl. During this period water distilled through the air into the KCl filling the tips of the electrodes. As soon as this process of distillation had raised the level of fluid in the tips sufficiently the electrodes were entirely filled with 3MKCl by means of a fine glass pipette. Complete immersion in 3MKCl for 24 hrs allowed the electrodes to become totally equilibrated and they were then ready for use. Electrodes had tip resistances between 10 and 30 MΩ. This parameter was measured using an internal circuit contained within the recording electrometer. Tip potentials were less than -3mV. Microelectrodes were washed in distilled water after removal from the KCl storage solution prior to an experiment.

2. Non-polarisable electrodes.

Non-polarizable (reversible) electrodes were of the calomel cell or Ag : AgCl type.

Before being plated with silver chloride, silver wires were cleaned thoroughly with fine emery paper and de-greased (petrol ether). The indifferent electrode was held as close as possible to the wires to be plated to ensure maximum plating current density. The electrolyte was 0.1N HCl. Current was supplied by a 2V accumulator. Initially the wires intended for subsequent use as non-polarizable electrodes were connected to the anode. After 30 secs the current was reversed and this cycle was repeated three times. The electrodes were ready for use after washing in distilled water.

A chlorided silver wire inserted directly into the end of a KCl filled microelectrode formed its connection to the recording apparatus. Care was taken to ensure that bubbles were not introduced into the end of the microelectrode by this procedure. The reference electrode consisted of a glass tube filled with KCl : Agar and connected to the recording apparatus by a second chlorided silver wire. In some preliminary experiments (not shown) a calomel cell, placed at the periphery of the culture dish, formed the indifferent electrode. This arrangement was not favoured since the resulting asymmetry of electrode potentials could result in a small current being passed across the membrane of the impaled cell thus altering the resting potential.

3. Microscope, micromanipulator and screened cage.

The cultured cells were viewed using a Prior inverted microscope (Code No. 383) fitted with a long working range condenser, and the

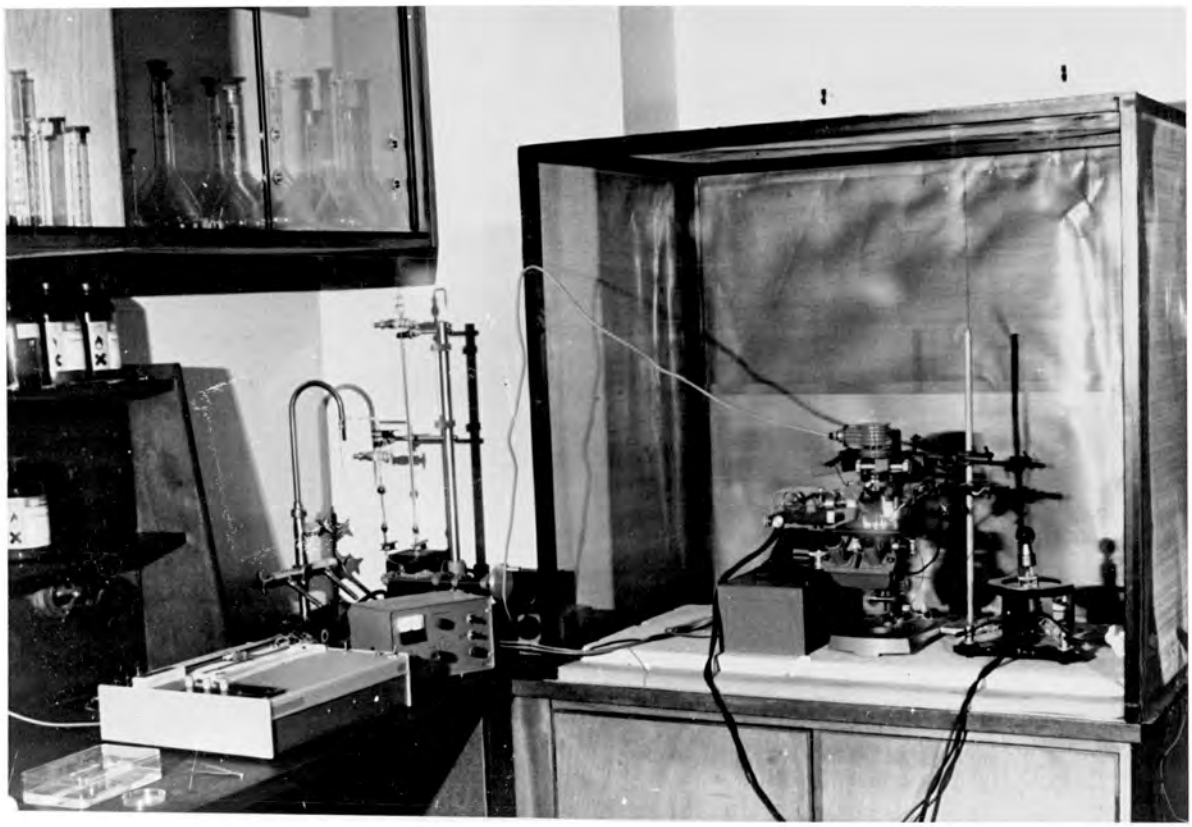

PLATE 7

Apparatus employed for the measurement of membrane potentials in cultured cells.

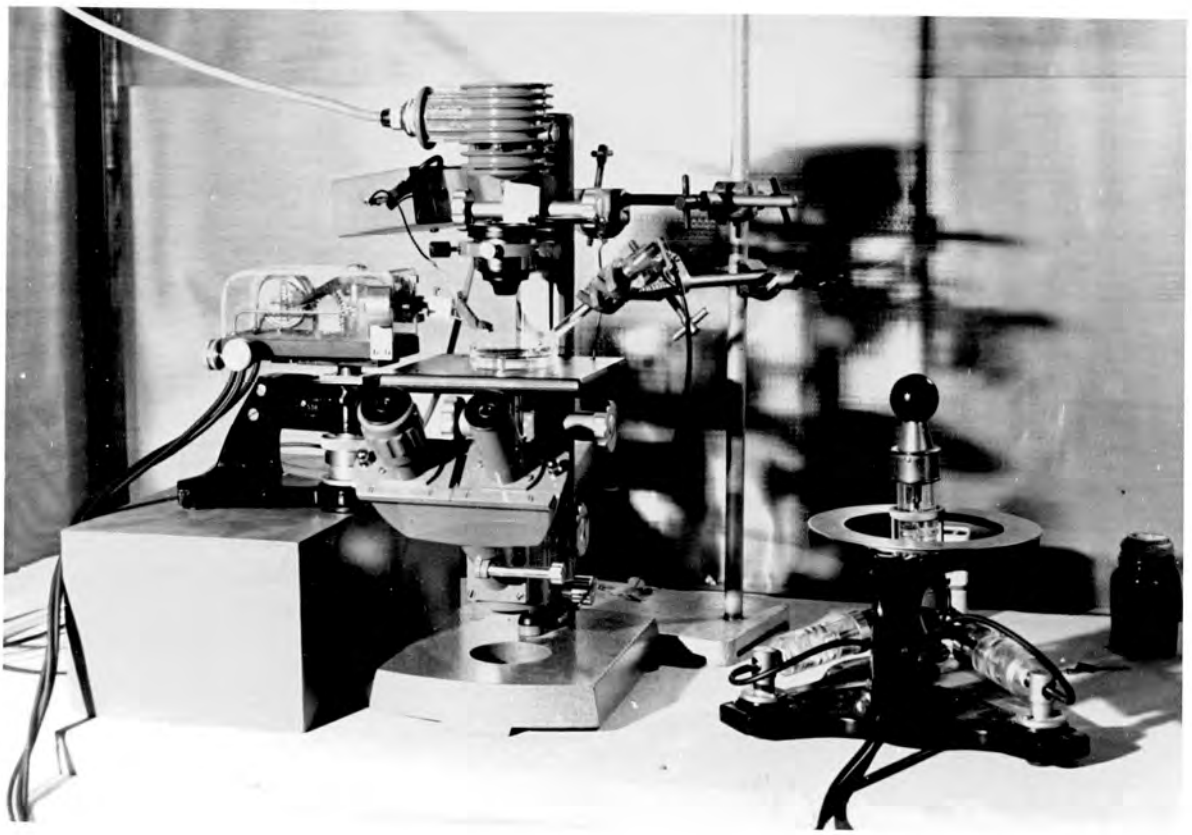

PLATE 8

Close up view of apparatus housed within the screened cage.

microelectrodes controlled by a De Fonbrone pneumatic micro-manipulator. This apparatus was housed in a screened cage (PLATES 7 & 8).

4. Recording apparatus.

The microelectrodes were connected to the input of a Medistor Electrometer Amplifier (Model A35). Potentials were amplified by the Medistor equipment and recorded on a Servoscribe potentiometric recorder. Tracings were calibrated by comparison with standard voltage pulse (10 or 50 mV) provided by the electrometer.

5. Preparation of cultures and microelectrode technique.

Primary cultures of acinar cells were grown on hydrated collagen lattices in McCoy's 5A + 20% calf serum. The innoculation density was 5×10^5 cells/ml and the growth time 5 days. At this stage the monolayer was not confluent, neither were the epithelial cells overgrown by fibroblasts. Since it is known that membrane potentials of cultured cells vary according to the cell density and growth phase (PEACOCK, MINNA, NELSON & NIRENBERG, 1972) the same innoculation density and growth time were adhered to throughout.

Intracellular potentials were recorded only from epithelial cells. A suitable colony was identified and the cells impaled under direct visual control. Care was taken to ensure that no cell was impaled more than once. Three criteria were required to be fulfilled before a potential recording was judged as acceptable:-

(1) A rapid negative deflection as the cell was impaled.

(2) A decay time longer than 10 secs.

(3) A rapid return of the record to zero potential upon with-

drawing the electrode from the cell. Inevitably the application of such criteria leads to some selection of the results.

A considerable amount of practice was required before cells could be successfully impaled. Of particular importance was the angle at which the microelectrode approached the cell to be impaled - an angle of about 45° to the collagen substratum proving the most satisfactory. A large number of failures was probably due to the microelectrode passing completely through the thin cultured cell and into the underlying collagen. Advancing the electrode directly into the collagen resulted in a large positive potential, due to bending of the microelectrole tip, suggesting that the negative membrane potentials were not artifacts derived from this source.

Most experiments were performed in Hanks B.S.S., the cultures being washed several times in this solution prior to starting. This medium was chosen for convenience since its low bicarbonate concentration (4.2 mM) does not necessitate equilibration with CO_2 rich gas mixtures to maintain a stable physiological pH. In one experiment Eagle's MEM + 20% calf serum was employed. All experiments were performed at room temperature ($21-23^{\circ}C$). The effects of CCK-Pz, carbamylcholine and secretin were tested by measuring the potentials of a random control population of epithelial cells within a given culture and comparing these with another population from the same dish measured in the presence of the hormones. The cultures were then washed three times with Hanks B.S.S. and a second control group of potentials measured. Most experiments performed lasted between $1\frac{1}{2}$ and 2 hrs. The duration of exposure to secretagogues was not the same in all experiments and tended to depend on the ease with which membrane potentials were being recorded from that particular culture. It was

not possible to hold the potentials long enough to determine the effect of stimulants on a single cell.

Results are expressed as the mean ± standard error of mean (number of observations). Statistical significance was calculated using the student t test.

RESULTS

1. Resting Membrane Potential.

A total of 246 epithelial cell membrane potentials were measured in Hanks B.S.S. at the start of 6 separate experiments. In these experiments the mean resting potential varied between 5.7 ± 0.5 mV and 8.4 ± 0.3 mV inside negative. The mean potential for all cells impaled was -7.4 ± 0.2 mV (246). Fig. 31 shows the frequency distribution for this population and the Gaussian distribution calculated from the histogram values after applying Sheppards correction. The histogram shows a skewdness towards the higher potentials but probably indicates that one population of cells was being impaled.

In one experiment in which potentials were measured in Eagle's MEM + 20% calf serum the mean resting potential was -10.8 ± 0.7 mV (12). This was significantly different ($P < 0.001$) from potentials measured in Hanks B.S.S.

Examples of recordings are shown in Fig. 32. Only potentials whose contour complied with the criteria stated in Methods were included in the results analysis. From an initial rapid negative deflection the potentials demonstrated variable rates of decay, probably due to ion leakage at the site of impalement. The commonest profiles recorded were those illustrated in B and C with a fairly rapid decay from the initial peak. Occasionally potentials were recorded that stabilised at or near the initial peak level e.g. A. For analysis purposes the height of the initial rapid negative deflection was taken to represent the intracellular potential. The longest time for which a stable potential was held was 70 sec., usually the potential decayed or the microelectrode dislodged much sooner. This was the factor that

prevented testing the effect of secretagogues on a single cell. Normally, provided the potential was maintained for at least 10 sec the microelectrode was withdrawn manually. This resulted in a sharp positive deflection of the recording and a return to the base-line level. A,B,C,D & E. (Fig. 32)

2. Effect of increasing extracellular K^+

The effect of elevating the extracellular K^+ was tested in one experiment (Fig. 33). The mean resting potential in Hanks B.S.S. K^+ = 5.8 mEq/l was 5.7 ± 0.5 mV (20). Increasing the extracellular K^+ to 15 and 20 mEq/l caused a fall in the mean potential to 4.9 ± 0.3mV (25) and 4.5 ± 0.5 mV (14) respectively. These values however were not statistically significant from the controls $P > 0.2$. In the intact mouse pancreas increasing the extracellular K^+ to 20mM results in a fall of membrane potential from -39.2 to -23.8 mV (MATTHEWS & PETERSEN 1973).

3. Effect of CCK-Pz

The effect of CCK-Pz (5 C.H.R. units/ml) was tested in 3 experiments. In all of these CCK-Pz caused a statistically significant depolarization of the membrane potential.

Fig. 34: 8.0 ± 0.5 mV (18) to 5.0 ± 0.4 mV (25) $p < 0.001$.

Fig. 36: 10.8 ± 0.7 mV (12) to 8.4 ± 0.5 mV (13) $p < 0.05$

Fig. 35: 8.4 ± 0.3 mV (57) to 5.5 ± 0.6 mV (33) $p < 0.001$

Washing the cultures with Hank's B.S.S. after exposure to CCK-Pz resulted, in two of these experiments in an increase in mean membrane potential.

Fig. 34: 5.0 ± 0.4 mV (25) to 11.5 ± 0.6 mV (12) $p < 0.001$

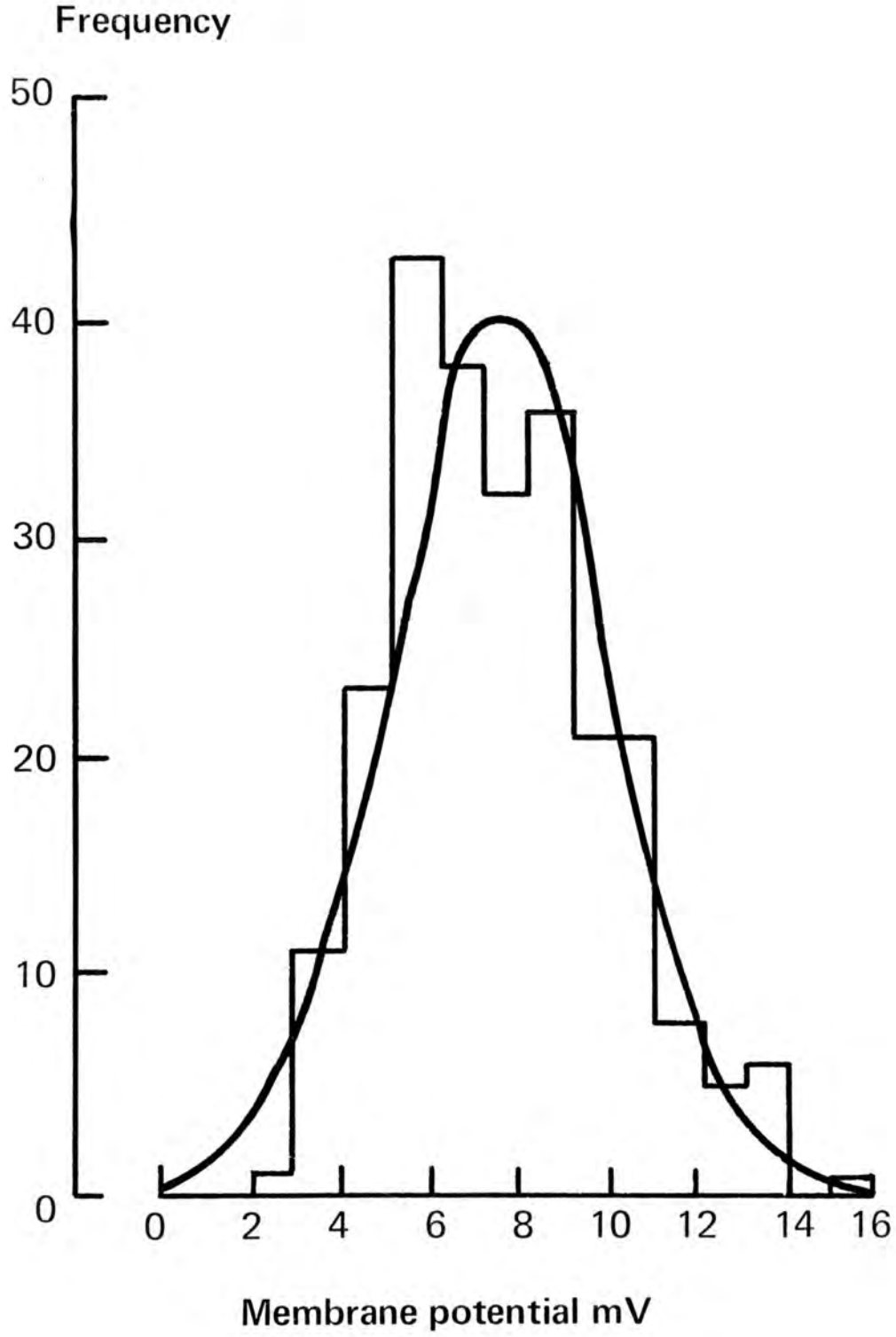

FIG. 31

Frequency distribution histogram for 246 epithelial cell resting membrane potentials (6 experiments). The Gaussian distribution for the population was calculated from the histogram values after applying Sheppards correction.

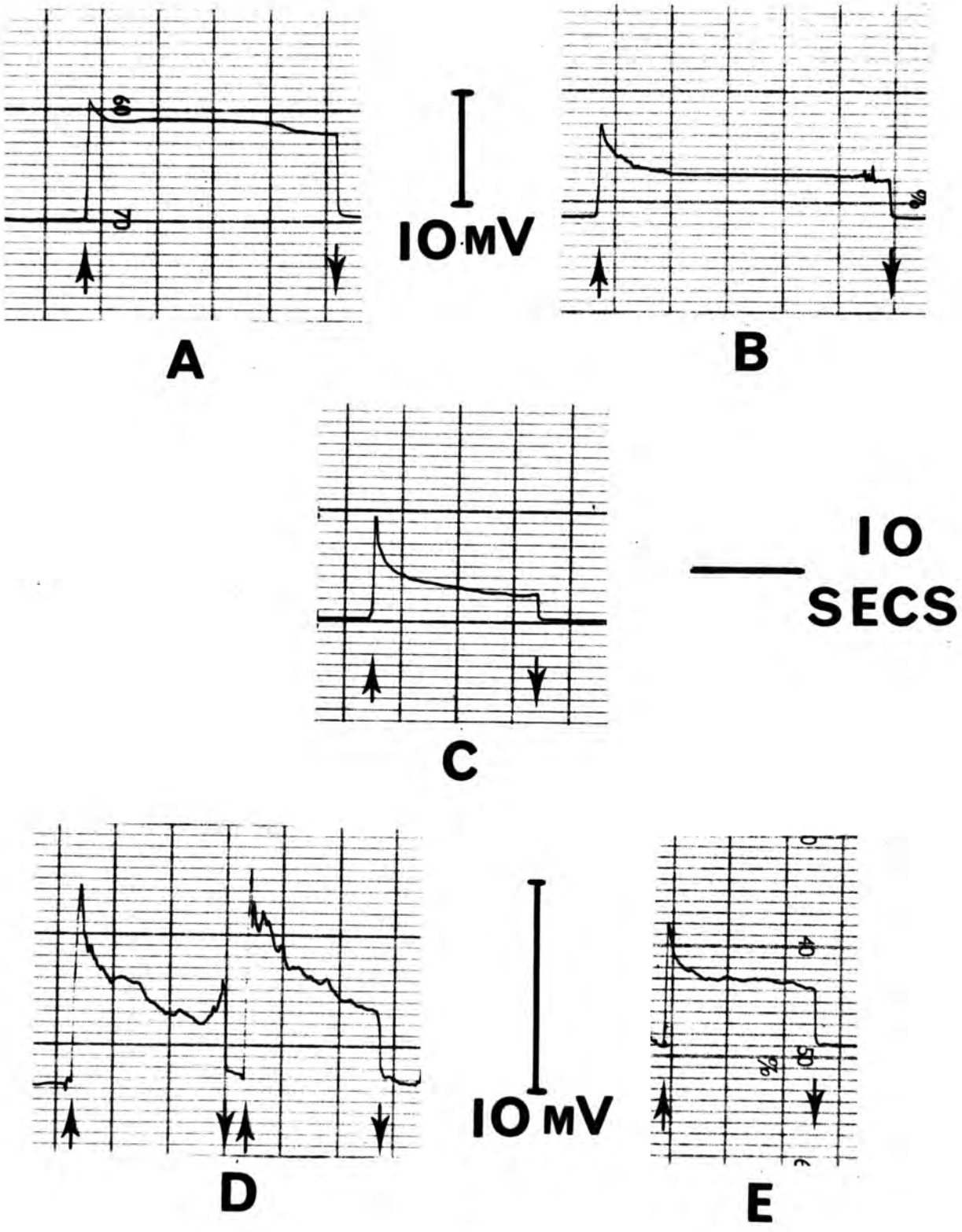

FIG. 32

Membrane potentials recorded from epithelial cells in 5 day primary cultures of guinea pig cells. An upward deflection represents a negative potential. The arrows indicate the times at which the microelectrode was advanced into ↑ and withdrawn from ↓ a cell.

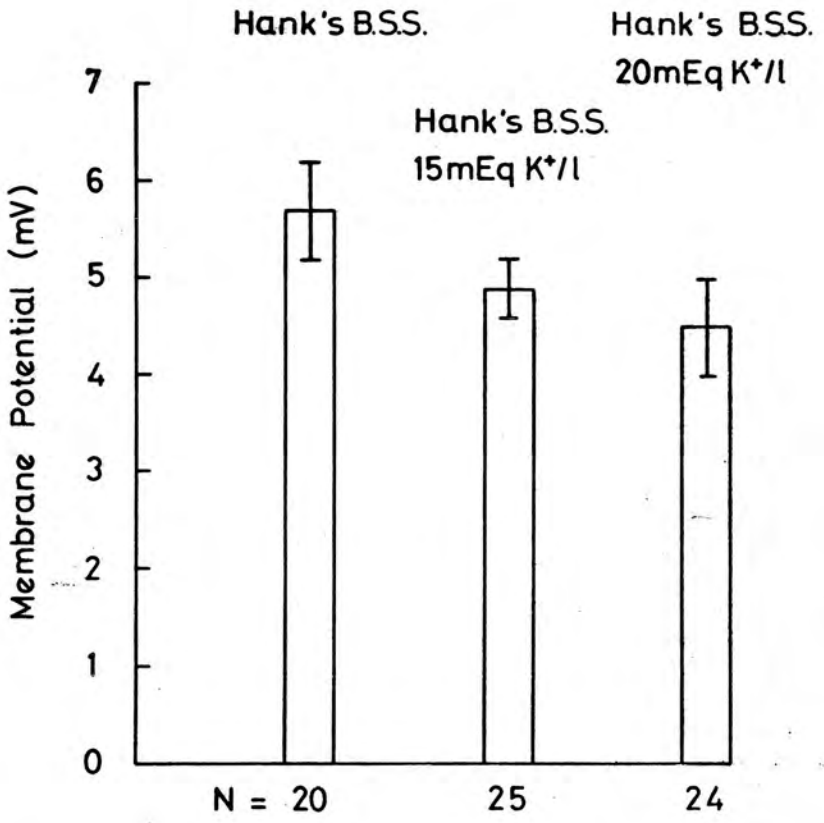

FIG. 33

The effect of elevating the extracellular potassium ion concentration on membrane potentials recorded from epithelial cells. 5 day primary culture of guinea pig pancreatic cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Hank's B.S.S. at room temperature. Sufficient KCl was dissolved in the Hank's B.S.S. to give the required final concentration. One experiment.

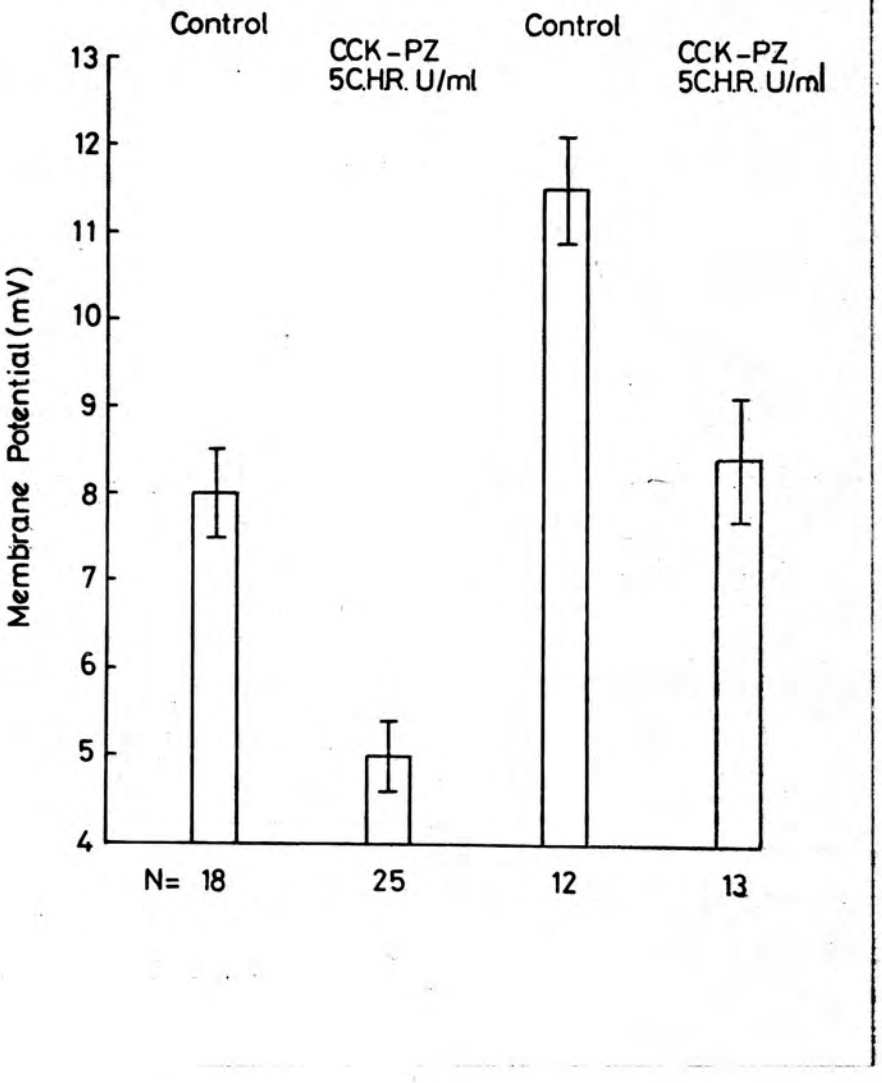

FIG. 34

The effect of cholecystokinin Pancreozymin (CCK-Pz) on the membrane potential of epithelial cells. This experiment shows that the depolarizing effect of CCK-Pz (5 C.H.R. U/ml) is repeatable in a single experiment. 5 day primary culture of guinea-pig pancreatic cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Hank's B.S.S. at room temperature. One experiment.

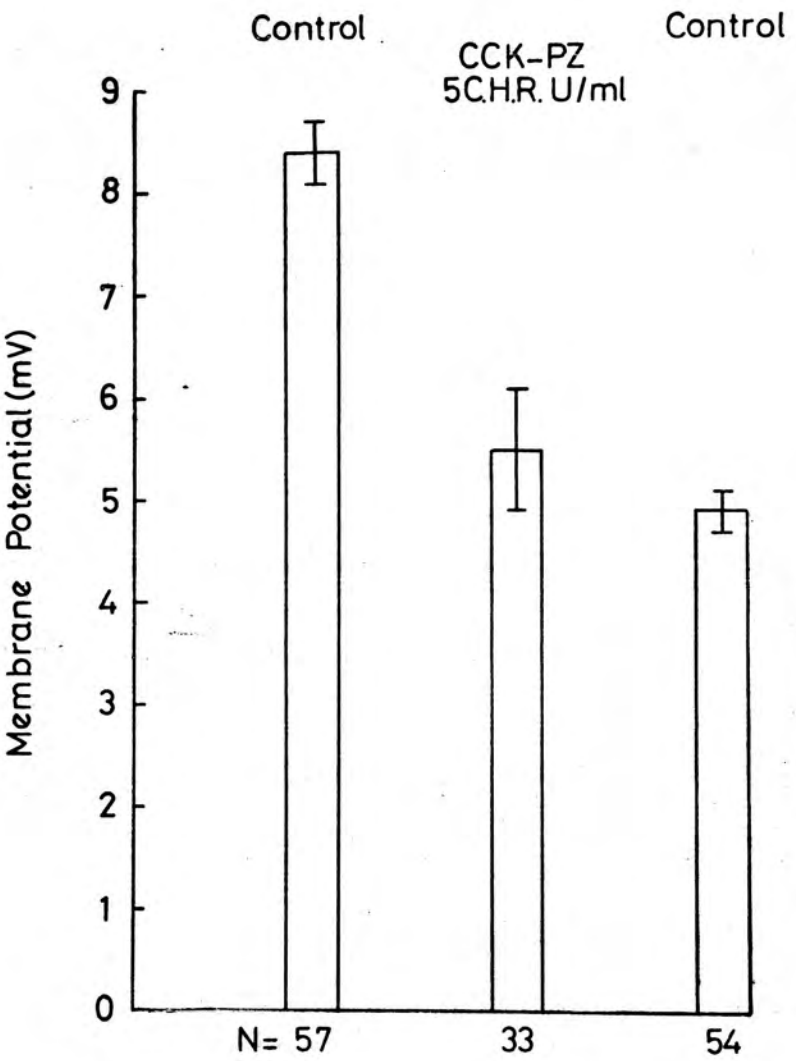

FIG. 35

The effect of cholecystokinin-pancreozymin (CCK-Pz) 5 C.H.R.U./ml on the membrane potential of epithelial cells. 5 day primary culture of guinea-pig cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Hank's B.S.S. at room temperature. One experiment.

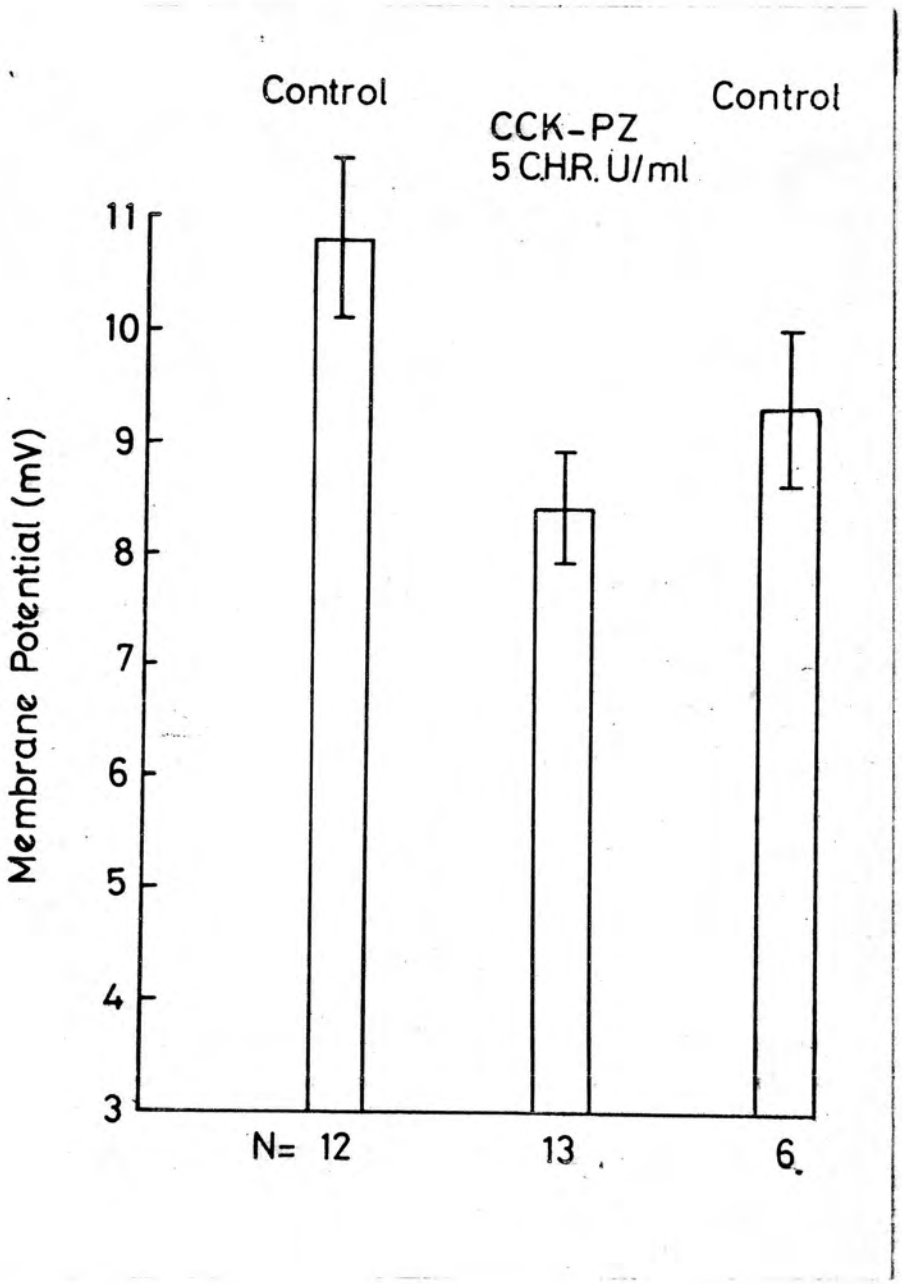

FIG. 36

The effect of cholecystokinin-pancreozymin (CCK-Pz) 5 C.H.R.U./ml on the membrane potential of epithelial cells. 5 day primary culture of guinea-pig pancreatic cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Eagle's M.E.M. + 20% calf serum at room temperature. One experiment.

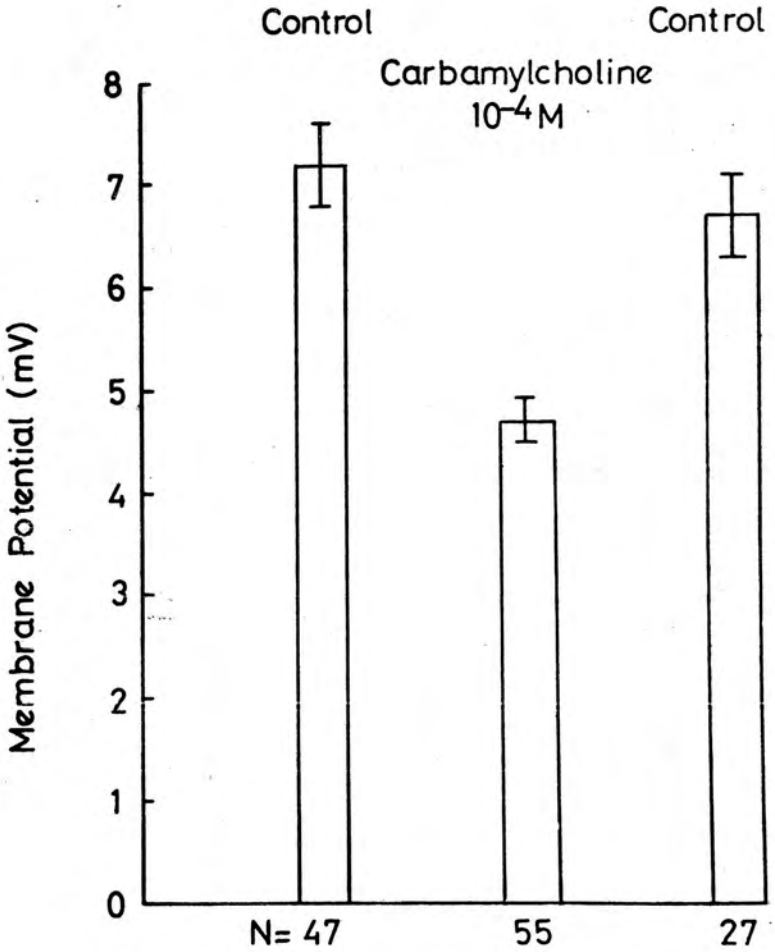

FIG. 37

The effect of carbamylcholine (10^{-4}M) on the membrane potential of epithelial cells. 5 day primary culture of guinea-pig pancreatic cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Hank's B.S.S. at room temperature. One experiment.

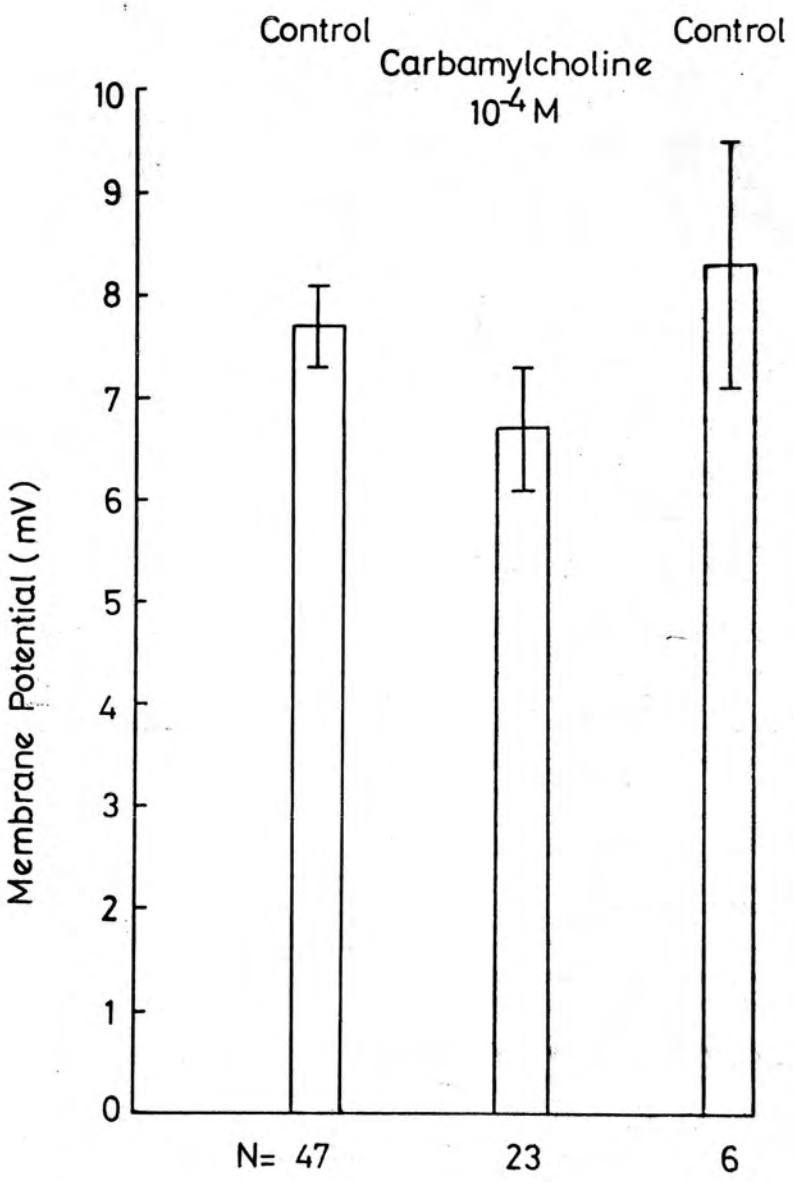

FIG. 38

The effect of carbamylcholine (10^{-4}M) on the membrane potential of epithelial cells. 5 day primary culture of guinea pig pancreatic cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Hank's B.S.S. at room temperature. One experiment.

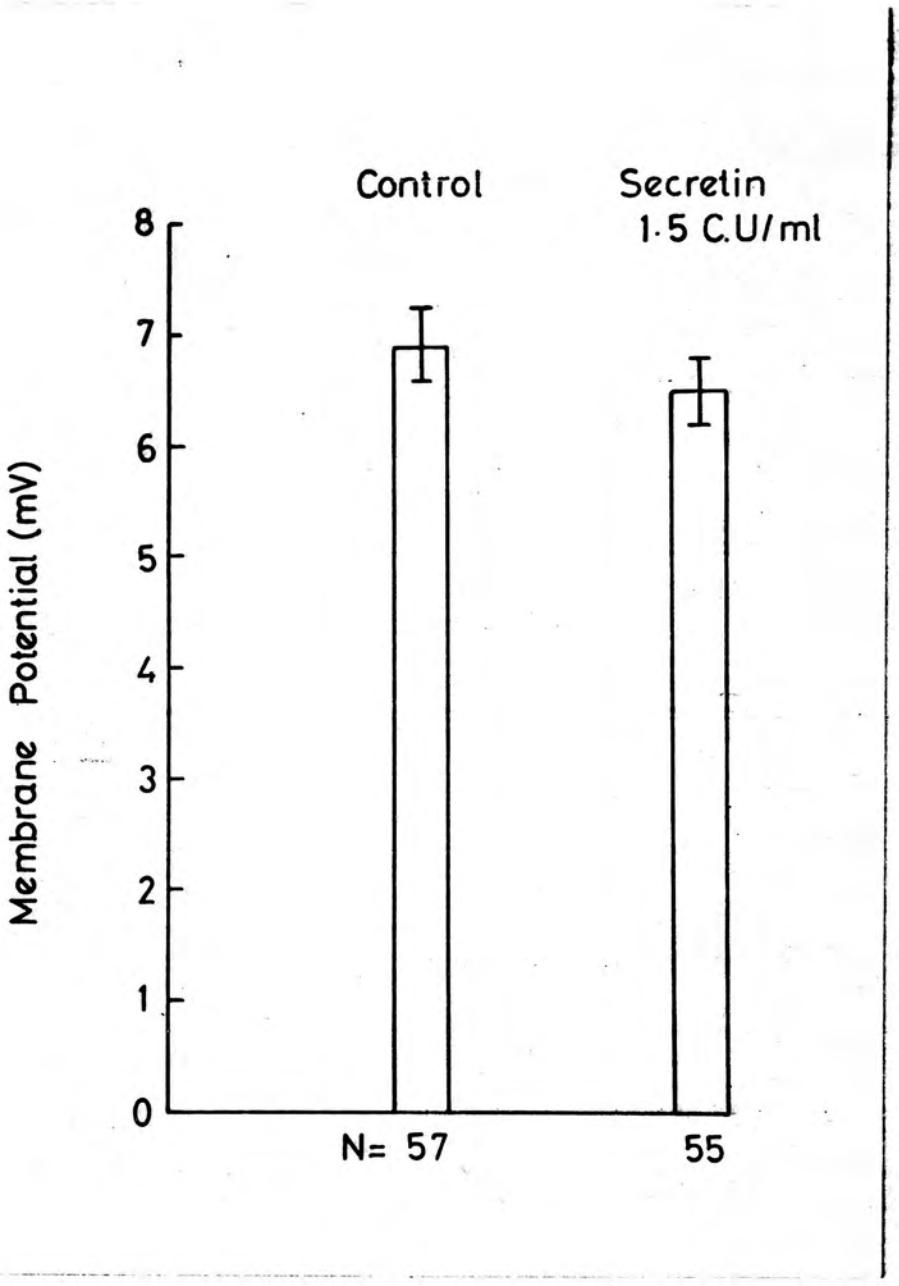

FIG. 39

The effect of secretin (1.5 C.U./ml) on the membrane potential of epithelial cells. 5 day primary culture of guinea pig pancreatic cells grown on hydrated collagen lattices. The growth medium was McCoy's 5A + 20% calf serum and the potentials were measured in Hank's B.S.S. at room temperature. One experiment.

Fig. 36: 8.4 ± 0.5 mV (13) to 9.3 ± 0.7 mV (6) $p \geqslant 0.4$.

This effect was statistically significant in one experiment (fig. 34) and reflected a hyperpolarization of the potential compared to the previously recorded resting level. Furthermore in this experiment a second exposure to CCK-Pz caused a statistically significant fall in potential from 11.5 ± 0.6 mV (12) to 8.4 ± 0.7 mV (13) $p < 0.01$ demonstrating that the depolarizing effect of the hormone was repeatable.

4. Effect of Carbamylcholine.

In 2 experiments carbamylcholine (10^{-4}M) caused a fall in the mean membrane potential.

Fig. 37: 7.2 ± 0.4 mV (47) to 4.7 ± 0.2 mV (55) $p < 0.001$.

Fig. 38: 7.7 ± 0.4 mV (47) to 6.7 ± 0.6 mV (23) $p \geqslant 0.2$

This depolarization was statistically significant in one experiment (Fig. 37). Reversal of this effect could be achieved by washing the cultures with Hank's B.S.S.

Fig. 37: 4.7 ± 0.2 mV (55) to 6.2 ± 0.4 mV (27) $p < 0.001$

Fig. 38: 6.7 ± 0.6 mV (23) to 8.3 ± 1.2 mV (6) $p \geqslant 0.4$

Again this was statistically significant in only one experiment (Fig. 37). The effect of atropine on this carbamylcholine induced depolarization was not tested.

5. Effect of Secretin.

In one experiment secretin (1.5 CU/ml) caused a small fall in mean membrane potential.

Fig. 39: 6.9 ± 0.3 mV (57) to 6.5 ± 0.3 mV (55)

This effect was not statistically significant $p \geqslant 0.4$ and was smaller

in magnitude than any of the effects observed with the stimulants of enzyme secretion.

DISCUSSION

1. Identity of the cells impaled.

Care was taken to ensure that only epithelial cells were impaled a procedure that was facilitated by the fact that the experiments were performed under direct visual control. The origin of the epithelial cells however remains open to question and the possibility exists that they were derived from either acinar, insular or duct cells all of which were present in the original cell suspensions used for setting up the cultures. Phase contrast microscopy did not reveal any criteria for the separation of the descendants of these different cell types within the epithelial population. Biochemical and morphological evidence suggested that the acinar cells undergo dedifferentiation *in vitro* losing their capacity to synthesise and secrete digestive enzymes. However since stereological studies have shown that acinar cells comprise 82% of the total glandular volume in the guinea-pig (BOLENDER 1974) and since cell suspensions obtained from the gland were at least 90% acinar it seems reasonable to conclude that most of the epithelial cells in the cultures were descendants, albeit despecialised descendants of this cell type. Any other conclusion would have to assume that insular and duct cells have much higher rates of division *in vitro*. There is however no evidence within the literature or within the present study to suggest this. This conclusion is in agreement with the results of a careful study by ORCI et al. (1973) on monolayer cultures of newborn rat pancreas. Histological and electronmicroscope techniques were employed to follow the fate of the insular and acinar cells. Their conclusion (discussed in Part III)

was that the acinar cells become despecialised and that their descendants, represented as dedifferentiated epithelial cells, were present with a great frequency in all cultures beyond 2.5 days of age. Taken together these pieces of evidence suggest that the random sampling employed in these electrophysiological experiments does result in mostly acinar cell potentials being recorded simply because the descendants of these cells are present in the greatest numbers. The probability that some records were made from other cell types cannot be excluded but providing the ratio of acinar to other epithelial cells was high and providing sufficient numbers of cells were sampled their inclusion should not significantly affect the results. Confirmation that this is the case comes from the frequency distribution of the resting potentials recorded at the start of the experiments. This distribution (Fig. 31), mode 5-6mV, although skewed towards higher potentials suggests that only one population of cells were being sampled. Some of the skewdness may have been introduced by the criteria imposed for acceptance of a potential (see Methods) since higher potentials were often more stable with decay times well in excess of the 10 sec minimum. The relationship between the stability of membrane potentials and their level for in vivo epithelial tissue has been studied in detail by FROMTER, MULLER & WICK (1971). Some positive skewdness could also be introduced by variation in electrode tip potentials, however HÜLSER (1974) has determined that tip potentials decrease when the microelectrode enters a cultured cell creating a tendency for the cell potential to be underestimated.

2. Resting Membrane Potential.

The resting membrane potential of the cultured epithelial cells was always negative with respect to the bathing medium and averaged -7.4 ± 0.2 mV (246). This is much lower than acinar cell potentials recorded from a variety of *in vitro* preparations of the intact gland, mouse, -41.2 ± 1.4 mV (DEAN & MATTHEWS, 1972) Cat -33.8 ± 1.0 mV, rabbit -54.9 ± 1.3 mV (NISHIYAMA & PETERSEN 1974) rat -35 ± 0.5 mV (KANNO 1972). Taking into account the difficulties encountered with cell impalement (principally due to the thinness ($\simeq$ 0.2 μ) of the cultured cells) the possibility exists that the present results are artifacts introduced by the experimental technique and not meaningful records of the intracellular potentials. There is no clear cut answer to this question; however several points should be considered. The waveforms recorded were characteristic of intracellular potentials - a rapid negative deflection on impalement followed by a variable rate of decay and a rapid positive deflection on withdrawal of the electrode. The potentials were always negative. Deliberate impalement of the substratum caused a large positive deflection suggesting that the potentials were not artifacts arising from this source. Impalement of a cell did not result in any morphological changes characteristic of cell death i.e. rounding up or vesiculation. Experiments in which secretagogues were tested showed alterations in the membrane potential in the same direction as those most commonly observed in the intact gland. On the other hand secretin which has no effect on the acinar cell potential *in situ* (DEAN & MATTHEWS 1972) also had no significant effect on the cultured cells. Finally the same apparatus and experimental technique when used to study other cells in culture e.g. myoblasts, muscle cells

and fibroblasts resulted in the recording of higher potentials (M.O. WRIGHT 1975 personal communication).

These observations tend to suggest that the recordings did represent intracellular potentials. The problem remains as to why these were of such low magnitude compared to those recorded from the intact tissue. One possibility is that impalement caused partial damage to the cells, this is evidenced by the potential decay after the initial rapid negative deflection. Although there was no evidence that impalement caused cell death the decay could be attributed to poor sealing of the plasma membrane around the microelectrode. In a study on cultured chick embryo heart DEHAAN & GOTTLIEB (1968) found that the success with which they impaled cells depended on three factors: microelectrode resistance, constitution of the culture medium and the oxygen tension. Their best recordings were obtained using ultramicroelectrodes with tip resistances between 100 and 150 MΩ a medium containing embryo extract and cultures incubated for 48 hrs in a gas atmosphere containing 10% oxygen. In addition LEHKUHL & SPERELAKIS (1963) have also shown improved sealing with an increased extracellular calcium concentration, a reduction in pH from 7.4 to 7.2 and in the absence of phenol red.

Another possible cause of the low potentials is the fact that all experiments were performed at room temperature. A consequent decrease in the rate of intracellular chemical reactions would be expected to dissipate cation gradients across the plasma membrane and consequently diminish the resting potential. Lowering the temperature of mammalian muscle from 37 to 22°C results in a fall of 5 to 10 mV in the resting membrane potential (BOYD & MARTIN, 1959). Only two studies have included investigations of the effect of temperature on membrane

potentials in cultured cells. The results are however contradictory. BORLE & LOVEDAY (1968) measured the membrane potential of Hela cells and found that this fell from -11.9 to -9.2 mV when the temperature was lowered from 27 to 17°C. Conversely HUSLER (1971) has reported that for a number of different cell types in culture a change in temperature from 37 to 22°C had no significant effects on membrane potentials.

HÜLSER (1971) has however observed that the HCO_3^- concentration of the medium affects the magnitude of the membrane potential. Reductions from 44 to 6mM (pH maintained constant) caused falls in potential of 20 mV in 3T3 and BICR/MIRK cells and of 5mV in Hela and KB cells. The present studies were performed in Hank's B.S.S. (HCO_3^- = 4.2 mM) while the cells were grown in McCoy's 5A which contains a higher HCO_3^- concentration (26 mM). Thus transference for the electrophysiological studies resulted in a reduction in the HCO_3^- of the medium surrounding the cells.

Apart from technical considerations the very fact that cultured cells are grown in an artificial environment may cause alterations in potential and make comparisons with the same cell <u>in situ</u> difficult. CONE (1971a, b) has been foremost in advancing the proposal that cells undergoing mitosis have low resting potentials and has suggested that the level of the membrane potential or more exactly the intracellular ionic conditions which it reflects may act to control mitosis. In support of this theory he claims that cultured cells with high membrane potentials are usually those which undergo infrequent or no division. That this is generally true can be seen from Table IV which represents a survey of membrane potentials recorded from a variety of different cultured cells. Those which

do not divide in culture i.e. neurones and mature muscle cells generally have higher potentials. Individual examples can be cited - the potential of chick myotubules varies between -5 and -80mV being dependent on the size and maturity of the tubules, the greater the maturity and size the higher the potential (FISCHBACH, NAKEROFF & NELSON, 1971). Transformation of cells to the malignant state in vivo is accompanied by a lowering of the membrane potential e.g. myosarcoma cells -10mV and adjacent non-dividing muscle -90mV (BALITSKY & SHUBA, 1964). The membrane potentials of hamster CHO cells increase from -15mV to -55mV as confluence approaches (CONE, 1971a), and those of neuroblastoma cells vary according to the growth phase being lowest in log and highest in stationary (PEACOCK et al. 1972). Since the pancreatic cell cultures employed in this study were 5 days of age and had not reached confluency this additional factor may go towards explaining the low membrane potentials recorded in this study compared with those from acinar cells in the intact gland.

3. The effect of extracellular K^+

Increasing the extracellular potassium concentration of the medium bathing the pancreatic cell cultures caused a small but statistically insignificant fall in the resting membrane potential. Similar results have been obtained by ANUL (1967) for Ehrlich Ascites tumour cells and by NELSON, PEACOCK & MINNA (1972) for L cells. In the former study measurement of intracellular ion concentrations and transmembrane fluxes indicated that the resting membrane potential (-11.2 mV) was about one third of the equilibrium potential for chloride ions. This difference was due to a high permeability to sodium (P_{Na}). Analagous conclusions have also been reached by LAMB &

McKINNON (1971) for L cells. Flux data indicated similar permeabilities to Na^+ and K^+ and a high permeability to Cl^-. The P_{Na}/P_K ratio was 0.67 and these workers concluded that the low membrane potential (-15mV) was due to a low absolute P_K rather than a high P_{Na}. The same may hold for pancreatic acinar cells in culture.

The membrane potential of acinar cells *in situ* is not solely determined by the K^+ distribution. Elevation of extracellular potassium does cause depolarization (KANNO, 1972; MATTHEWS & PETERSEN, 1973). When the K_{out} is greater than 10 mM each 10 fold change in concentration produces a 28 mV change in membrane potential (MATTHEWS & PETERSEN, 1973). This is less than the 61mV predicted by the Nernst equation for a cell, i.e. nerve or muscle freely and predominantly permeable to potassium ions. Given that the intracellular cation concentrations are similar to those in excitable cells (SCHNEYER & SCHNEYER, 1960) it can be concluded that there must be a difference in ion permeabilities. Calculation of the P_{Na}/P_K ratio for many non-excitable cells shows this to be larger than for nerve and muscle and would account for the reduced slope of potential vs. Log K_{out} plots commonly observed (WILLIAMS, 1970).

4. Effect of CCK-Pz, carbamylcholine and secretin on the membrane potential.

In the intact gland CCK-Pz, Ach, acetyl-β-methylcholine and stimulation of the pancreatic nerves causes a fall in the resting membrane potential of about 15mV (DEAN & MATTHEWS, 1972; PETERSEN & MATTHEWS, 1972;; MATTHEWS & PETERSEN, 1973; MATTHEWS, PETERSEN & WILLIAMS, 1973; NISHIYAMA & PETERSEN, 1974; GREENWELL, 1975). In one study (KANNO, 1972) secretory stimulants hyperpolarized acinar cells

by approximately 5mV.

Most of the experiments in which depolarizing responses have been observed were performed under conditions in which the gland was exposed to secretory stimulants for only a short time. This procedure produces a reversible, short lasting depolarization. Contradictory results have been obtained when exposure to the stimulant has been prolonged. GREENWELL (1975) observed that the response was always transitory even in the continued presence of the stimulant - the membrane potential returning to its resting level in 10 mins whereas MATTHEWS & PETERSEN (1973) have reported that following prolonged exposure (20-30 min) depolarization is maintained but recovery to the resting potential is slow. Since in this study potentials were measured from a population of cells over a period of many minutes it would appear that the depolarizing effect observed was probably also long lasting in the cultured cells. This effect of CCK-Pz and carbamylcholine was reversible. Withdrawal of the secretagogue resulted in an increase in the mean membrane potential in 4 out of 5 experiments although statistical significance was reached in only two instances. In one of these experiments a second exposure to CCK-Pz caused a statistically significant depolarization. These results suggest that the effects were not artefacts due to deterioration of the preparation during the course of an experiment. Thus although the acinar cells lose their ability to synthesise and secrete digestive enzymes when cultivated in vitro the preliminary work presented here suggests that they retain at least at the level of the cell membrane, one component of the physiological response to stimulation. Since the dedifferentiation is only partial the possibility exists that improvement in the culture techniques could

result in the propagation of fully functional acinar type cells.

Secretin had no significant effects on the membrane potential. In the intact gland secretin causes a long lasting hyperpolarization of the duct cells (GREENWELL, 1975) but has no effect on the membrane potential of the acinar cells (DEAN & MATTHEWS, 1972; GREENWELL, 1975).

BIBLIOGRAPHY

AGREN, G. & LAGERLOF, H. (1936). The pancreatic secretion in man after intravenous administration of secretin. Acta med. Scand. 90, 1-29

de ALMEIDA, A.L. & GROSSMAN, M.I. (1952). Experimental production of pancreatitis with ethionine. Gastroenterology 20, 554-577.

ALONSO , G.L., BAZERQUE, P.M., ARRIGO, D.M. & TUMILASCI, O.R. (1971). Adenosine triphosphate dependent calcium uptake by rat submaxillary gland microsomes. J. Gen. Physiol. 58, 340-350.

AMSTERDAM, A. & JAMIESON, J.D. (1972). Structural and functional characterization of isolated pancreatic exocrine cells. Proc. Nat. Acad. Sci. U.S.A. 69, 3028-3032.

AMSTERDAM, A. & JAMIESON, J.D. (1974a). Studies on dispersed pancreatic exocrine cells 1. Dissociation technique and morphologic characteristics of separated cells. J. Cell. Biol. 63, 1037-1056.

AMSTERDAM, A. & JAMIESON, J.D. (1974b). Studies on dispersed pancreatic exocrine cells. II Functional characteristics of separated cells. J. Cell. Biol. 63, 1057-1073.

ANDERSON, N.G. (1953). The mass isolation of whole cells from rat liver. Science 117, 627.

ANREP, G.V. (1916). The influence of the vagus on pancreatic secretion. J. Physiol. 50, 421-433.

ANUL, F. (1967). Measurement of the electrical potential difference across the membrane of the Ehrlich mouse Ascites tumour cell. J. Cell. Physiol. 69, 21-32.

ARGENT, B.E., SMITH, R., & CASE, R.M. (1975). Biochem. J. (in the press)

AYUSO, M.S., FISHER, A.B., PARILLA, R. & WILLIAMSON, J.R. (1973). Glucose metabolism by isolated rat lung cells. Am. J. Physiol. 225, 1153-1160.

BABKIN, B.P. (1924). The influence of the blood supply on pancreatic secretion. J. Physiol. 59, 153-163.

BALITSKY, K.P. & SHUBA, E.P. (1964). Resting potential of malignant tumour cells. Acta. Unio. int. Contra. Canr. 20, 1391-1393.

BARRINGTON, E.J.W. (1941). The influence of secretin on pancreatic secretion in the cat. J. Physiol. 100, 80-93.

BASS, R.L. & SALTMAN, P. (1959). The accumulation of iron by rat liver cell suspensions. Exptl. Cell. Res. 18, 560-572.

BAYLISS, W.M. & STARLING, E.H. (1902). The mechanism of pancreatic secretion. J. Physiol. 28, 325-353.

BDOLAH, A., BEN-?VI, R. & SCHRAMM, M. (1964). The mechanism of enzyme secretion by the cell. II. Secretion of amylase and other proteins by slices of rat parotid gland. Arch. Biochem. Biophys. 104, 58-66.

BECKER, V. (1962). Histochemistry of the exocrine pancreas. In Ciba. Found. Symp. The exocrine pancreas, ed. de Reuck, A.V.S. & Cameron, M.P. London: Churchill.

BEN-OR, S. & DOLJANSKI, F. (1960). Single cell suspensions as tissue antigens. Exptl. Cell. Res. 20, 641-644.

BERG, N.B. & YOUNG, R.W. (1971). Sulphate metabolism in pancreatic acinar cells. J. Cell Biol. 50, 469-483.

BERNARD, C. (1849). Du suc pancréatique et de son rôle dans les phénomenès de la digestion. Arch. gén. Méd., Ser. 4, 19, 60

BERNARD, C. (1856). Memoire sur le pancreas et sur le rôle due suc pancréatique dans le phénomenès digestifs, particulierement dans la digestion des matières grasses neutres. C.r. hebd. Seanc. Acad. Sci., Paris Suppl. 1, 379.

BERRY, M.N. (1962). Metabolic properties of cells isolated from adult mouse liver. J. Biol. Chem. 15, 1-8.

BERRY, M.N. & FRIEND, D.S. (1969). High yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell. Biol. 43, 506-520

BERRY, M.N. & SIMPSON, F.O. (1962). Fine structure of cells isolated from adult mouse liver. J. Biol. Chem. 15, 9-17.

BEŽNAK, A.B.L. (1934). On the mechanism of the autacoid function of parasympathetic nerves. J. Physiol. 82, 129-153.

BIRKS, R.I. & COHEN, M.W. (1968). The action of sodium pump inhibitors on neuromuscular transmission. Proc. Roy. Soc. B. 170, 381-399.

BLUM, A.L., HIRSCHOWITZ, B.L., HELANDER, H.F. & SACHS, G. (1971). Electrical properties of isolated cells of necturus gastric mucosa. Biochim et Biophys Acta. 241, 261-272.

BODANSZKY, M. (1973). The synthesis of (Porcine) Secretin. In Handbook of Experimental Pharmacology. ed. Jorpes, J.E. & Mutt V. Vol. XXXIV. pp. 180-194.

BOHM, J. (1931). Untersuchungen über Zweikernige Zellen II. Die Auszählung und Berechnung der zweikernigen heberzellen. Ztsch. f. mikr-Anat. Forsch 24, 263-285.

BOLENDER, R.P. (1974). Stereological analysis of the guinea pig pancreas. 1. Analytical model and quantitative description of non-stimulated pancreatic exocrine cells. J. Cell. Biol. 61, 269-287.

BORLE, A.B. & LOVEDAY, J. (1968). Effects of temperature, potassium and calcium on the electrical potential difference in Hela cells. Cancer Research. 28, 2401-2405.

BOYD, I.A. & MARTIN, A.R. (1959). Membrane constants of mammalian muscle fibres. J. Physiol. 147, 450-457.

BRANSTER, M.V. & MORTON, R.K. (1957). Isolation of intact liver cells. Nature 180, 1283-1284.

BRAY, G.A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1, 279-285.

BROWN, G.L. & FELDBERG, W. (1936). The action of potassium on the superior cervical ganglion of the cat. J. Physiol. 86, 290-305.

BROWN, J.C., HARPER, A.A. & SCRATCHERD, T. (1967). Potentiation of secretin stimulation of the pancreas. J. Physiol. 190, 519-530.

CALDWELL, P.C. & DOWNING, A.C. (1955). The preparation of capillary microelectrodes. J. Physiol. 128, 31P.

CASE, R.M. & CLAUSEN, T. (1971a). Calcium ions and the release of enzymes from rat pancreas in response to gastrointestinal hormones. Acta endocr. (Kbh.) Suppl. 155, 203.

CASE, R.M. & CLAUSEN, T. (1971b). Stimulus-secretion coupling in exocrine pancreas. II. The role of calcium ions. In Alfred Benzon Publication I. Gastrointestinal Hormones and other Subjects, ed. Thaysen, E.H. pp. 62-66. Copenhagen: Munksgaard.

CASE, R.M. & CLAUSEN, T. (1973). The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. J. Physiol. 235, 75-102.

CASE, R.M., HARPER, A.A. & SCRATCHERD, T. (1968). Water and electrolyte secretion by the perfused pancreas of the cat. J. Physiol. 196, 133-149.

CASE, R.M., HARPER, A.A. & SCRATCHERD, T. (1969a). The secretion of electrolytes and enzymes by the pancreas of the anaesthetised cat. J. Physiol. 201, 335-348.

CASE, R.M., HARPER, A.A. & SCRATCHERD, T. (1969b). Water and electrolyte secretion by the pancreas. In The Exocrine Glands, ed. Botelho, S.Y., Brooks, F.P. & Shelly, W.B. pp. 39-60. Philadelphia: University of Pennsylvania Press.

CASE, R.M., SCRATCHERD, T. & WYNNE, R. D'A. (1970). The origin and secretion of pancreatic juice bicarbonate. J. Physiol. 210, 1-15.

CHEN, J.M. (1954). The cultivation in fluid medium of organised liver, pancreas and other tissues of foetal rats. Exptl. Cell. Res. 7, 518-529.

CONE, C.D. (1971a). Unified theory on the basic mechanism of normal mitotic control and oncogenesis. J. Theor. Biol. 30, 151-181.

CONE, C.D. (1971b). Maintenance of mitotic homeostasis in somatic cell populations. J. Theor. Biol. 30, 183-194.

COUPLAND, R.E. (1958). The innervation of pancreas of the rat, cat and rabbit as revealed by the cholinesterase technique. J. Anat. 92, 143-149.

COVELL, W.P. (1928). A microscopic study of pancreatic secretion in the living animal. Anat. Rec. 40, 213-223.

CRAWFORD, A.C. & FETTIPLACE, R. (1971). A method for altering intracellular calcium concentrations. J. Physiol. 217, 20-21P.

CRICK, J., HARPER, A.A. & RAPER, H.S. (1949). On the preparation of secretin and pancreozymin. J. Physiol. 111, 367-376.

CUATRECASAS, P. (1972). Perturbation of the insulin receptor of isolated fat cells with proteolytic enzymes. Direct measurement of insulin receptor interactions. J. Biol. Chem. 246, 6522-6531.

CUATRACASAS, P. (1973). Insulin receptor of liver and fat cell membranes. Fed. Proc. 32, 1838-1846.

DEAN, P.M. & MATTHEWS, E.K. (1972). Pancreatic acinar cells. Measurement of membrane potential and miniature depolarization potentials. J. Physiol. 225, 1-13.

DEHAAN, R.L. & GOTTLIEB, S.H. (1968). The electrical activity of embryonic chick heart cells isolated in tissue culture singly or in interconnected cell sheets. J. Gen. Physiol. 52, 643-665.

DIAMOND, J.M., BARRY, P.H. & WRIGHT, E.M. (1971). The route of transepithelial ion permeation in the gall-bladder. In Symposia Medica Hoechst, Electrophysiology of Epithelial Cells, pp. 22-38. Stuttgart-New York: Schattauer Verlag.

DOLINSKY, I.L. (1894). Influence of acids on the secretion of the pancreatic gland. (RUSSIAN). Arch. Sci. biol. St. Petersb. 3, 399. Cited by Babkin, B.P. in Secretory Mechanisms of the digestive glands, 2nd ed. 1950. p. 563. New York: Hoeber.

DOUGLAS, W.W. (1968). Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Br. J. Pharmacol. 34, 451-474.

DOUGLAS, W.W. & KANNO, T. (1967). The effect of amethocaine on acetylcholine-induced depolarization and catecholamine secretion in the adrenal chromaffin cell. Br. J. Pharmacol. 30, 612-619.

DOUGLAS, W.W., KANNO, T. & SAMPSON, S.R. (1967). Influence of the ionic environment on the membrane potential of adrenal chromaffin cells and on the depolarizing effect of acetylcholine. J. Physiol. 191, 107-121.

DOUGLAS, W.W. & POISNER, A.M. (1963). The influence of calcium on the secretory response of the submaxillary gland to acetylcholine or to noradrenaline. J. Physiol. 165, 528-541.

DOUGLAS, W.W. & POISNER, A.M. (1964). Stimulus-secretion coupling in a neurosecretory organ: the role of calcium in the release of vasopressin from the neurohypophysis. J. Physiol. 172, 1-18.

DOUGLAS, W.W. & RUBIN, R.P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol. 159, 40-57.

DOUGLAS, W.W. & RUBIN, R.P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. J. Physiol. 167, 288-310.

DREILING, D.A., JANOWITZ, H.D. & HALPERN, M. (1955). The effect of a cationic anhydrone inhibitor, Diamox, on human pancreatic secretion. Gastroenterology 29, 262-279.

DREISBACH, R.H. (1964). Regulation of calcium transfer in rat salivary and lacrimal glands in vitro. Amer. J. Physiol. 207, 1015-1020.

DREISBACH, R.H. (1967). Effect of isoproterenol on calcium, protein and electrolytes of rat submaxillary gland. Proc. Soc. Exp. Biol., N.Y. 126, 279-281.

DUNCAN, P.R., HARPER, A.A., HOWAT, H.T., OLEESKY, S., VARLEY, H. (1950). The effects of pancreozymin on human subjects. J. Physiol. 111, 63P.

EKHOLM, R., ZELANDER, T. & EDLUND, Y. (1962). The ultrastructural organization of the rat exocrine pancreas. II. Centroacinar cells, intercalary and intralobular ducts. J. Ultrastructure. Res. 7, 73-83.

ELLIOT, K.A.C. & LIBET, B. (1942). Studies on the metabolism of brain suspensions. I. Oxygen Uptake. J. Biol. Chem. 143, 227-246.

ELSDALE, T. & BARD, J. (1972). Collagen substrata for studies on cell behaviour. J. Cell. Biol. 54, 626-637.

EXTON, J.H. (1964). Metabolism of rat liver cell suspensions 1. General properties of isolated cells and occurrence of the citric acid cycle. Biochem. J. 92, 457-467.

FELDBERG, W. & GUIMARAIS, J.A. (1936). The liberation of acetylcholine by potassium. J. Physiol. 86, 306-314.

FENN, W.O. (1940). The role of potassium in physiological processes. Physiol. Rev. 20, 377-415.

FINDLAY, J.A., GILL, J.R., LEVER, J.D., RANDLE, P.J. & SPRIGGS, T.L.B. (1969). Increased insulin output following stimulation of the vagal supply to the perfused rabbit pancreas. J. Anat. 104, 580.

FISCHBACH, G.D., NAMEROFF, M. & NELSON, P.G. (1971). Electrical properties of chick skeletal muscle fibres developing in cell culture. J. Cell. Physiol. 78, 289-300.

FLATLAND, R.F., SCHNEYER, L.H. & SCHNEYER, C.A. (1969). Amylase Activity of Acinar Cells Separated from Guinea Pig Submaxillary Gland. Proc. Soc. Exp. Biol. & Med. 181, 243-246.

FORTE, J.G. & NAUSS, A.H. (1963). Effects of calcium removal on bullfrog gastric mucosa. Am. J. Physiol. 205, 631-637.

FROMTER, E., MULLER, C.W. & WICK, T. (1971) Permeability properties of the proximal tubular epithelium of the rat kidney studied with electrophysiological techniques. In Electrophysiology of Epithelial Cells. Ed. Giebisch, G. Stuttgart New York, Shattauer Verlag pp. 119-148.

GIBBONS, J.L. & RIENITZ, K.G. (1961). Diphosphopyridine nucleotide and the respiration of rat-liver-cell suspensions. Biochim. Biophys. Acta. 47, 395-397.

GOEBELL, H., STEFFAN, C., BALTZER, G. & BODE, C. (1973). Stimulation of pancreatic secretion of enzymes by acute hypercalcemia in man. Eur. J. clin. Invest. 3, 98-104.

GOEBELL, H., STEFFAN, C. & BODE, C. (1972). Stimulatory effect of pancreozymin-cholecystokinin on calcium secretion in pancreatic juice of dogs. Gut 13, 477-482.

GOSPODAROWICZ, D. & GOSPODAROWICZ, F. (1972). A technique for the isolation of bovine luteal cells and its application to metabolic studies of luteal cells in vitro. Endocrinology. 90, 1427-1434.

de GRAAF, R. (1664). De natura et usu succi pancreatici. Translated by Pack, C., 1676. London: Brook.

GREENWELL, J.R. (1975). The effects of cholecystokinin-Pancreozymin, acetylcholine and secretin on the membrane potentials of mouse pancreatic cells in vitro. Pflugers Arch. 353, 159-170.

GREGORY, H., HARDY, P.M., JONES, D.S., KENNER, G.W. & SHEPPARD, R.C. (1964). The antral hormone gastrin. Nature 204, 931-933.

GRODSKY, G.M. & BENNETT, L.L. (1966). Cation requirements for insulin secretion in the isolated perfused pancreas. Diabetes, 15, 910-913.

GROSSMAN, M.I. & IVY, A.C. (1946). Effect of Alloxan upon external secretion of the pancreas. Proc. Soc. Exp. Biol. & Med. N.Y. 63, 62-63.

GUIDOTTI, G.C., LUNEBERG, B. & BORGHETTI, F. (1969). Amino acid uptake in chick embryo heart cells. Effect of insulin. Biochem. J. 114, 97-105.

GWATKIN, R.L.B. & THOMSON, J.L. (1964). A new method for dispersing the cells of mammalian tissues. Nature. 201, 1242-1243.

GWYNN, J.A.P., JONES, B., JONES, B.M. & KEMP, R.B. (1970). A modified perfusion technique for preparing isolated liver cells. An ultrastructural study. Cytobios. 2, 181-191.

HALES, C.N. & MILNER, R.D.G. (1968). The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol. 194, 725-743.

HANKS, J.H. & WALLACE, J.H. (1958). Determination of cell viability. Proc. Soc. Expl. Biol. Med. 98, 188-192.

HARPER, A.A. & RAPER, H.S. (1943). Pancreozymin, a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine. J. Physiol. 102,115-125.

HARPER, A.A. & VASS, C.C.N. (1941). The control of the external secretion of the pancreas in cats. J. Physiol. 99, 415-435.

HEIDENHAIN, R. (1868). Beiträge zur Lehre von der Speichelabsonderung. Stud. physiol. Inst. Breslau. Heft 4. p. 1 & 30. Cited by Babkin, B.P. in Secretory mechanisms of the digestive glands, 2nd ed. 1950, p.23. New York: Hoeber.

HEIDENHAIN, R. (1875). Beiträge zur Kenntnis de Pankreas. Pflug. Arch. ges Physiol. 10 557. Cited by Foster, M. in A text book of physiology, 3rd ed. 1879, p.251. London: MacMillan.

HENLEY, K.S., SORENSEN, O. & POLLARD, H.M. (1959). Some enzymic properties of suspensions of parenchymatous liver cells. Nature 184, 1400.

HILWIG, I. & SCHUSTER, S. (1970. Uber das Wachstum der Pankreaszellen von Säugetieren als Monolayer Cultures II Bildung histotypischer Aggregate aus Suspensionen fetaler Menschlicher Pankreas zellen. Z. Zellforsch. 103, 282-290.

HILWIG, I., SCHUSTER, S., HEPTNER, W. & WASIELEWSKI, E. VON. (1968). Uber das Wachstum der Pankreaszellen von Säugetieren als monolayer cultures. I. Züchtungsmethode, Morphologie und Insulingehalt. Z. Zellforsch. 90, 333-346.

HILWIG, I. & VRBANEC, S. (1970) Uber das Wachstum der Pankreaszellen von Säugetielen als Monolayer Cultures III Beeinflussung der Insulinsekretion durch Coffein und Glucose. Z. Zellforsch. 103, 410-419.

HOKIN, L.E. (1966). Effects of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. Biochim. Biophys. Acta 115, 219-221.

HOWARD, R.B., CHRISTENSEN, A.K., GIBBS, F.A. & PESCH, L.A. (1967). The enzymic preparation of isolated intact parenchymal cells from rat liver. J. Cell. Biol. 35, 675-684.

HÜLSER, D.F. (1971). Elektrophysiologische untersuchungen an saugerzellkulturen: Der einfluß von Bicarbonat und pH auf das Membranpotential. Pflügers Arch. 325, 174-187.

HÜLSER, D.F. (1974). Ionic coupling between non-excitable cells in Culture. In Methods in Cell Biology Vol. VIII Ed. D.M. Prescott. Academic Press. New York & London. p.289-317.

ICHIKAWA, A. (1965). Fine structural changes in response to hormonal stimulation of the perfused canine pancreas. J. Cell. Biol. 24, 369-385.

IVY, A.C. & OLDBERG, E. (1928). A hormone mechanism for gall-bladder contraction and evacuation. Am. J. Physiol. 86, 599-613.

IYPE, P.I. & BHARGAVA, P.M. (1965). The respiration of isolated rat hepatic cells in suspension. Biochem. J. 94, 284-288.

JAANUS, S.D., ROSENSTEIN, M.J. & RUBIN, R.P. (1970). On the mode of action of ACTH on the isolated perfused adrenal gland. J. Physiol. 209, 539-556.

JACOB, S.T. & BHARGAVA, P.M. (1962). A new method for the preparation of liver cell suspensions. Exptl. Cell. Res. 27 453-467.

JACOBSON, A., SCHWARTZ, M. & REHM, W.S. (1965). Effects of removal of calcium from bathing media on frog stomach. Am. J. Physiol. 209, 134-140.

JAMIESON, J.D. & PALADE, G.E. (1967a). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell Biol. 34, 577-596.

JAMIESON, J.D. & PALADE, G.E. (1967b). Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. 34, 587-615.

JAMIESON, J.D. & PALADE, G.E. (1971a). Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: metabolic studies. J. Cell. Biol. 48, 503-522.

JAMIESON, J.D. & PALADE, G.E. (1971b). Synthesis, intracellular transport and discharge of secretory proteins in stimulated pancreatic exocrine cells. J. Cell. Biol. 50, 135-158.

JEŻYK, P.F. & LIBERTI, J.P. (1969). Metabolic activities of mechanically and enzymatically prepared rat liver cells. Arch. Biochem. Biophys. 134, 442-449.

JORPES, J.E. (1968). The isolation and chemistry of secretin and cholecystokinin. Gastroenterology. 55, 157-164.

JUTISZ, M. & PALOMA de la LLOSA, M. (1970). Requirement of Ca^{++} and Mg^{++} ions for the *in vitro* release of follicle-stimulating hormone from rat pituitary glands and in its subsequent biosynthesis. Endocrinology 86, 761-768.

KALANT, H. & YOUNG, F.G. (1957). Metabolic behaviour of isolated liver and kidney cells. Nature. 179, 816-817.

KALSER, M.H. & GROSSMAN, M.I. (1954). Pancreatic secretion in dogs with ethionine induced pancreatitis. Gastroenterology 26, 189-197.

KALTENBACH, J.P. (1952). Preparation of suspensions of intact liver cells. Fed. Proc. 11, 237.

KANNO, T. (1972). Calcium dependent amylase release and electrophysiological measurements in cells of the pancreas. J. Physiol. 226, 353-371.

KIMMICH, G.A. (1970). Active sugar accumulation by isolated intestinal epithelial cells. A new model for sodium dependent metabolic transport. Biochemistry. 9, 3669-3677.

KLOPPENBERG, P.W.C., ISLAND, D.P., LIDDLE, G.W., MICHELAKIS, A.M. & NICHOLSON, W.D. (1968). A method of preparing adrenal cell suspensions and its applicability to the in vitro study of adrenal metabolism. Endocrinology. 82, 1053-1058.

KOELLE, G.B. (1970). The Pharmacological Basis of Therapeutics. Ed L.S. Goodman & A. Gilman 4th Edn. p.467. New York. Macmillan.

KONO, T. (1969). Destruction and restoration of the insulin effector system of isolated fat cells. J. Biol. Chem. 244, 5777-5784.

KUHN, W. & LEA, A.S. (1882). Beobachtungen über die Absonderung des Pankreas. Untersuchungen physiol. Inst. Heidelberg 2, 448. Cited by Babkin, B.P. in Secretory mechanisms of the digestive glands. 2nd ed. 1950, p.20. New York: Hoeber.

LACY, P.E., HOWELL, S.L., YOUNG, D.A. & FINK, C.J. (1968). New hypothesis of insulin secretion. Nature. 219, 1177-1179.

LAGERLOF, H.O. (1942). Pancreatic function and pancreatic disease studied by means of secretin. Stockholm: Norsted & Soner.

LAMB, J.F. & McKINNON, M.G.A. (1971). The membrane potential and permeabilities of the L cell membrane to Na, K and chloride. J. Physiol. 213, 683-689.

LAMBERT, A.E., BLONDEL, B., KANAZAWA, L.Y., ORCI, L. & RENOLD, A.E. (1972). Monolayer cell culture of neonatal rat pancreas: light microscopy and evidence for immunoreactive insulin synthesis and release. Endocrinology. 90, 239-248.

LAMBERT, A.E., JEANRENAUD, B., JUNOD, A. & RENOLD, A.E. (1969). Organ culture of fetal rat pancreas. II. Insulin release induced by amino and organic acids, by hormonal peptides, by cationic alterations of the medium and by other agents. Biochim. Biophys. Acta 184, 540-553.

LATA, G.F. & REINERTSON, J. (1957). Cholesterol synthesis by isolated rat liver cells. Nature. 179, 47-48.

LAWS, J.O. & STICKLAND, L.H. (1956). Metabolism of isolated liver cells. Nature. 178, 309-310.

LEHMKUHL, D. & SPERELAKIS, N. (1963). Transmembrane potentials of trypsin dispersed chick heart cells cultured *in vitro*. Am. J. Physiol. 205, 1213-1220.

LEURET, F. & LASSAIGNE, J.L. (1825). Recherches physiologiques et chimiques pour servir a l'histoire de la digestion. Paris: Huzard. Cited by Bostock, J. in Elementary systems of Physiology, 2nd ed. 1828. vol. 3, p.420. London: Baldwin & Cradock.

LIU, T.C. & GORSKI, J. (1971). Free cell suspensions from ovarian tissue: Preparation and steroidogenic response to gonadotrophins. Endocrinology. 88, 419-426.

LONGMUIR, I.S. & Ap REES, W.A. (1956). Preparation of cell suspensions from rat liver. Nature. 177, 997.

LOEWENSTEIN , W.R., SOCOLAR, S.J., HIGASHINO, S., KANNO, Y. & DAVIDSON, N. (1965). Intercellular communication: renal urinary bladder, sensory and salivary gland cells. Science. 149, 295-298.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

LUDWIG, C. (1851). Versuche über die Beihilfe de Nerven zur Speichelabsonderung. 2. rat. Med., N.F. 1 255. Cited by Babkin, B.P. in Secretory mechanisms of the digestive glands, 2nd ed. 1950. p.17. New York: Hoeber.

MACCHI, I.A. & BLAUSTEIN, E.H. (1969). Cytostructure and endocrine function of monolayer cultures of neonatal hamster pancreas. Endocrinology. 84, 208-216.

MacLEOD, R.M. & FONTHAM, E.H. (1970). Influence of ionic environment on the in vitro synthesis and release of pituitary hormones. Endocrinology 86, 863-869.

MADDEN, R.E. & BURK, D.J. (1961). Production of viable single cell suspensions from solid tumors. Natl. Canc. Inst. 27, 841-855.

MALAISSE-LEGAE, F. & MALAISSE, W.J. (1971). Stimulus-secretion coupling of glucose-induced insulin release. III. Uptake of ^{45}calcium by isolated islets of Langerhans. Endocrinology 88, 72-80.

MALAISSE, W., MALAISSE-LAGAE, F., WRIGHT, P.H. & ASHMORE, J. (1967). Effects of adrenergic and cholinergic agents upon insulin secretion *in vitro*. Endocrinology 80, 975-978.

MANERY, J.F. (1966). Effect of Ca ions on membranes. Fedn. Proc. 25, 1804-1810.

MANN, P.J.G., TENNENBAUM, M. & QUASTEL, J.H. (1939). Acetylcholine metabolism in the central nervous system. The effects of potassium and other cations on acetylcholine liberation. Biochem. J. 33, 822-835.

MARLISS, E.B., WOLLHEIM, C.B., BLONDEL, B., ORCI, L., LAMBERT, A.E., STAUFFACHER, W., LIKE, A.A. & RENOLD, A.E. (1973). Insulin and glucagon release from monolayer cell cultures of pancreas from new born rats. Europ. J. Clin. Invest. 3, 16-26.

MARTINEZ, J.R. & PETERSEN, O.H. (1972). The importance of extracellular calcium for acetylcholine-evoked salivary secretion. Experientia 28, 167-168.

MATEYKO, G.M. & KOPAC, M.J. (1963). Cytophysical studies on living normal and neoplastic cells. Ann. N.Y. Acad. Sci. 105, 183-285.

MATTHEWS, E.K. & PETERSEN, O.H. (1973). Pancreatic acinar cells: Ionic dependence of the membrane potential and acetylcholine-induced depolarization. J. Physiol. 231, 283-295.

MATTHEWS, E.K., PETERSEN, O.H. & WILLIAMS, J.A. (1973). Pancreatic acinar cells: Acetylcholine induced membrane depolarization, calcium efflux and amylase release. J. Physiol. 234, 689-701.

MATTHEWS, E.K. & SAFFRAN, M. (1967). Steroid production and membrane potential measurements in cells of the adrenal cortex. J. Physiol. 189, 149-161.

MELLANBY, J. (1925). The mechanism of pancreatic digestion - The function of secretin. J. Physiol. 60, 85-91.

MOORE, E.W. & MAKHLOUF, G.M. (1968). Calcium in normal human gastric juice. A four-component model with speculation on the relation of calcium to pepsin secretion. Gastroenterology 55, 465-480.

MOSCONA, A.C. & MOSCONA, H. (1952). The dissociation and aggregation of cells from organ rudiments of the early chick embryo. J. Anat. 86, 287-301.

MURRELL, L.R. (1966). Mammalian pancreatic islet tissue in organ culture 1. Methods of culture and *in vitro* histogenesis. Exptl. Cell. Res. 41, 350-364.

MUTT, V. & JORPES, J.E. (1967). Contemporary developments in the biochemistry of the gastrointestinal hormones. Recent Progress. Hormone Res. 23, 483-495.

NELSON, P.G., PEACOCK, J. & MINNA, J. (1972). An active electrical response in fibroblasts. J. Gen. Physiol. 60, 58-71.

NIELSEN, S.P. & PETERSEN, O.H. (1972). Transport of calcium in the perfused submandibular gland of the cat. J. Physiol. 223, 685-697.

NISHIYAMA, A. & PETERSEN, O.H. (1974). Pancreatic Acinar cells: Membrane potential and resistance change evoked by acetylcholine. J. Physiol. 238, 145-158.

O'BRIEN, R.D., ELDEFRAWI, M.E. & ELDEFRAWI, A.T. (1972). Isolation of acetylcholine receptors. Ann. Rev. Pharmacol. 12, 19-34.

OLMSTED, J.B. & BORISY, G.G. (1973). Microtubules. Ann. Rev. Biochem. 42, 507-540.

ONTKO, J.A. (1967). Chylomicron free fatty acid and ketone body metabolism of isolated cells and liver homogenates. Biochim. Biophys. Acta. 137, 13-22.

ORCI, L., LIKE, A.A., AMHERDT, M., BLONDEL, B., KANAZAWA, Y., MARLISS, E.B., LAMBERT, A.E., WOLLHEIM, C.B. & RENOLD, A.E. (1973). Monolayer cell culture of neonatal rat pancreas: An ultrastructural and biochemical study of functioning endocrine cells. J. Ultrastructure. Research, 43, 270-297.

PALADE, G.E. & CLAUDE, A. (1949). The nature of the golgi apparatus. I. Parallelism between intracellular myelin figures and golgi apparatus in somatic cells. J. Morphology. 85, 35-69.

PARSA, I. (1973). Pancreas acinar cell differentiation VIII. Effects of methionine on DNA synthesis of pancreas anlage in organ culture. Exptl. Cell. Res. 82, 466-468.

PARSA, I., MARSH, W.H. & FITZGERALD, P.J. (1969). Pancreas acinar cell differentiation. I. Morphologic and enzymic comparisons of embryo rat pancreas and pancreas anlage grown in organ culture. Am. J. Path. 57, 457-488.

PAUL, J. (1970). Cell and Tissue Culture, p.353. 4th ed. E. & S. Livingstone. Edinburgh & London.

PAVLOV, I.P. (1910). The work of the digestive glands. Translated by Thompson, W.H. 2nd ed. London: Griffin.

PEACOCK, J., MINNA, J., NELSON, P. & NIRENBERG, M. (1972). Use of Aminopterin in selecting electrically active neuroblastoma cells. Exp. Cell. Res. 73, 367-377.

PEARSE, A.G.E. & WELBOURN, R.B. (1973). The Apudomas. Brit. Jou. Hosp. Med. 10, 617-624.

PEKAS, J.G. (1971). Pancreatic incorporation of ^{65}Zn and histidine-^{14}C into secreted proteins of the pig. Am. J. Physiol. 220, 799-803.

PERRIS, A.D. (1966). Isolation of the epithelial cells of the rat small intestine. Can. J. Biochem. 44, 687-693.

PETERSEN, O.H. & MATTHEWS, E.K. (1972). The effect of pancreozymin and acetylcholine on the membrane potential of the pancreatic acinar cells. Experientia 28, 1037-1038.

PETERSEN, O.H. & NISHIYAMA, A. (1974). Pancreatic acinar cells: effect of local acetylcholine application on membrane potential and resistance. Communication to the VII meeting of the European Pancreatic Club. Dundee. Scotland.

POPIELSKI, L. (1901). Ueber das peripherische reflectorische NervenCentrum des Pankreas. Pflüg. Arch. ges. Physiol. 86. 215. Cited by Mutt. V. in Ark. Kemi 1960, 15 75.

PORTANOVA, R., SMITH, D.K. & SAYERS, G. (1970). A trypsin technique for the preparation of isolated rat anterior pituitary cells. Proc. Soc. Expl. Biol. Med. 133, 573-576.

RASMUSSEN, M. & TENENHOUSE, A. (1968). Cyclic adenosine monophosphate, Ca^{++}, and membranes. Proc. natl. Acad. Sci., U.S.A. 59, 1364-1370.

RIBET, A., FEDOU, R. & FREXINOS, J. (1969). Ultrastructure of the canine exocrine pancreas following continuous intravenous infusion of secretin and injection of pancreozymin. Digestion 2 145-143.

RIDDERSTAP, A.S. & BONTING, S.L. (1969). Enzyme secretion by the isolated rabbit pancreas: absence of a relation with the Na-K activated ATPase. Pflügers Arch. 313, 53-61.

RINALDINI, L.M.J. (1958). The isolation of living cells from animal tissues. Int. Rev. Cytol. VII. 587-647.

ROBBERECHT, P. & CHRISTOPHE, J. (1971). Secretion of hydrolases by perfused fragments of rat pancreas: effect of calcium. Am. J. Physiol. 220, 911-917.

RODBELL, M. (1964). Metabolism of isolated fat cells. J. Biol. Chem. 239, 375-380.

ROSE, S.P.R. (1967). Preparation of enriched fractions from cerebral cortex containing isolated, metabolically active neuronal and glial cells. Biochem. J. 102, 33-43.

ROUSE, P. & JONES, F.S. (1916). A method for obtaining suspensions of living cells from the fixed tissues, and for plating out of individual cells. J. Exp. Med. 23, 549-555.

RUBIN, R.P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22, 389-428.

RUBIN, R.P., FEINSTEIN, M.B., JAANUS, S.D. & PAIMRE, M. (1967). Inhibition of catecholamine secretion and calcium exchange in perfused cat adrenal glands by tetracaine and magnesium. J. Pharmacol. 155, 463-471.

SAMLI, M.H. & GESCHWIND, I.I. (1968). Some effects of energy-transfer inhibitors and of Ca^{++}-free or K^{+}-enhanced media on the release of luteinizing hormone (LH) from the rat pituitary gland in vitro. Endocrinology 82, 225-231.

SAYERS, G., SWALLOW, R.C. & GIORDANO, N.D. (1971). An improved technique for the preparation of isolated rat adrenal cells. A sensitive, accurate and specific method for the assay of ACTH. Endocrinology. 88, 1063-1068.

SCHEELE, G.A. & PALADE, G.E. (1975). Studies on the guinea pig pancreas. Parallel discharge of exocrine enzyme activities. J. Biol. Chem. (In the Press).

SCHNEIDER, W.C. & POTTER, U.R. (1943). The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem. 149, 217-227.

SCHNEYER, L.H. & SCHNEYER, C.A. (1960). Electrolyte and inulin spaces of rat salivary glands and pancreas. Am. J. Physiol. 199, 649-652.

SCHULZ, I. & MANNIGEL, H. (1974). Dependence of enzyme secretion on extracellular Ca^{++} and its modification by cyclic nucleotides and thyrocalcitonin. Communication to the VII meeting of the European Pancreatic Club. Dundee. Scotland.

SCHRAMM, M. (1967). Secretion of enzymes and other macromolecules. A. Rev. Biochem. 36, 307-320.

SCHRAMM, M. (1968). Amylase secretion in rat parotid slices by apparent activation of endogenous catecholamine. Biochem. Biophys. Acta 165, 546-549.

SEDAR, A.W. & FORTE, J.G. (1964). Effects of calcium depletion on the junctional complex between oxyntic cells of gastric glands. J. Cell Biol. 22, 173-188.

SELINGER, Z., EIMERL, S., SAVION, N. & SCHRAMM, M. (1974). A Ca^{++} ionophore A23187 stimulating hormone release and neurotransmitter action in the rat parotid and pancreas glands. In secretory mechanisms of Exocrine Glands ed. Thorn, N.A. & Petersen, O.H., pp. 68-87 Copenhagen: Munksgaard.

SELINGER, Z. & NAIM, E. (1970). The effect of calcium on amylase secretion by rat parotid slices. Biochim. Biophys. Acta 203, 335-337.

SELINGER, Z., NAIM, E. & LASSER, M. (1970). ATP Dependent calcium uptake by microsomal preparations from rat parotid and submaxillary glands. Biochim. Biophys. Acta. 203, 326-334.

SNEIDER, R.M. & PERSON, P. (1960). The isolation of submaxillary gland acini and duct segments. Exptl. Cell. Res. 20, 627-629.

St. AUBIN, P.M.G. & BUCHER, N.L.R. (1952). A study of binucleate cell counts in resting and regenerating rat liver employing a mechanical method for the separation of liver cells. Anat. Record. 112 797-809.

STEIN, E.A., HSIU, J. & RISCHER, E.H. (1964a). Alpha amylases as calcium metalloenzymes. I. Preparation of calcium-free apoamylases by chelation and electrodialysis. Biochemistry 3, 56-61.

STEIN, E.A., HSIU, J. & RISCHER, E.H. (1964b). Alpha amylases as calcium metalloenzymes. II. Calcium and the catalytic action. Biochemistry 3, 61-66.

SWALLOW, R.L. & SAYERS, G. (1969). A technique for the preparation of isolated rat adrenal cells. Proc. Soc. Expl. Biol. Med. 131, 1-4.

TAKEDA, Y., ICHIHARA, A., TANIOKA, H. & INOUE, H. (1964). The biochemistry of animal cells I. The effect of corticosteroids on leakage of enzymes from dispersed rat liver cells. J. Biol. Chem. 239, 3590-3596.

TIEDEMANN, F. & GMELIN, L. (1826). Die Verdauung nach Versuchen. Leipzig and Heidelberg. Groos. Cited by Bostock, J. in Elementary systems of physiology. 2nd ed. 1828, vol. 3. p.420. London: Baldwin & Cradock.

TONG, W. (1964). Stimulatory effect of thyrotropin on synthesis of thyroxine by isolated thyroid cells. Endocrinology. 74, 304-306.

VALE, W. & GUILLEMIN, R. (1967). Potassium-induced stimulation of thyrotropin release in vitro. Requirement for presence of calcium and inhibition by thyroxine. Experientia 23, 855-857.

VAN ZUTPHEN, H. (1970). Ph.D. Thesis. University of Utrecht.

VOGT, M. (1952). The secretion of the denervated adrenal medulla of the cat. Brit. J. Pharmacol. 7, 325-330.

WALLACH, D. & SCHRAMM, M. (1971). Calcium and the exportable protein in rat parotid gland. Parallel subcellular distribution and concomitant secretion. Eur. J. Biochem. 21, 433-437.

WESSELLS, N.K. (1964). DNA synthesis mitosis and dedifferentiation in pancreatic acinar cells *in vitro*. J. Cell. Biol. 20, 415-433.

WIEJPES, J.A. & PROP, F.J.A. (1970). Improved method for the preparation of single cell suspensions from mammary glands of adult virgin mouse. Exptl. Cell. Res. 61, 451-454.

WILLIAMS, J.A. (1970). Origin of transmembrane potentials in Non-excitable cells. J. theor. Biol. 28, 287-296.

WILLIAMS, J.A. & LEE, M. (1974). Pancreatic acinar cells: Use of the Ca^{++} ionophore to separate amylase release from the earlier steps in stimulus secretion coupling. Biochem. Biophys. Res. Comm. 60, 542-548.

WILLIAMS, J.A. & WOLFF, J. (1970). Possible role of microtubules in thyroid secretion. Proc. Nat. Acad. Sci. U.S.A. 67, 1901-1908.

WILLMER, E.N. (1960). Morphological problems of cell type, shape and identification. In Cells and Tissues in Culture. Methods Biology and Physiology Vol. I ed. Willmer E.N. pp. 143-174. Academic Press: London New York.

WIRSUNG, J.G. (1643). Cited by Foster, M. in lectures in the history of physiology, 1925, p.103. Cambridge: University Press.

ZIMMERMAN, M., DEVLIN, T.M. & PRUSS, M.P. (1960). Anaerobic glycolysis of dispersed cell suspensions from normal and malignant tissues. Nature. 185, 315-316.

ZIMMERMAN, M.J., DREILLING, D.A., ROSENBERG, I.R. & JANOWITZ, H.D. (1967). Secretion of calcium by the canine pancreas. Gastroenterology 52, 865-870.

ZIMMERMAN, M.J., MOORE, E.W., DREILLING, D.A. & JANOWITZ, H.D. (1971). Pancreatic juice ionized and total calcium in dogs in response to secretin, pancreozymin and calcium infusions. J. clin. Invest. 50, 103a.

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nulation of enzyme secretion from the perfused cat pancreas potassium

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is known that hormones can be released from the neurosecretory inals of the posterior pituitary gland (Douglas & Poisner, 1964) and the chromaffin cells of the adrenal medulla (Douglas & Rubin, 1961) by ng the extracellular potassium concentration. Furthermore, Bdolah, -Zvi & Schramm (1964) have shown that excess potassium stimulates me secretion from parotid gland slices. It was therefore decided to the effect of potassium on the pancreas, using a perfused preparation h we have recently described (Case, Harper & Scratcherd, 1968).

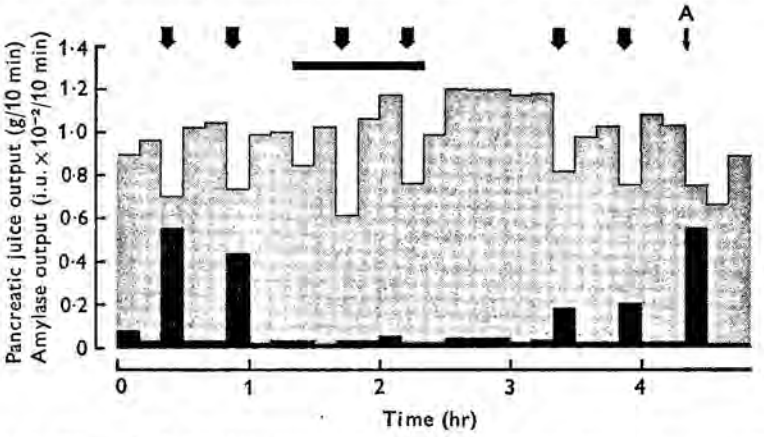

Fig. 1. The effect of atropine on the volume (stippled) and enzyme (in black) responses of the perfused pancreas to excess potassium. Secretion was maintained throughout by infusing secretin at a supra-maximal dose (8 μg/min). The perfusate contained atropine sulphate (7×10^{-7} M) for the length of the horizontal bar. The broad arrows indicate five minute periods during which the perfusate contained 50 mM-K^+. The final arrow (marked A) indicates a single injection of 5 μg acetylcholine.

agreement with Douglas & Poisner (1964), little effect was noticed potassium concentrations below 30 mM. However, higher concentions of potassium caused the pancreas to secrete copious amounts of me. Most of the enzyme appeared during the first few minutes of sure to excess potassium, though an elevated output was maintained ughout the test period. Accompanying the enzyme stimulation, ssium also caused a constant reduction in the volume of pancreatic tion.

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Two mechanisms can be suggested to explain this release of enzymes. Either potassium has a direct action on the pancreatic cell or it acts by depolarizing the cholinergic nerve terminals, thus causing release of acetylcholine with consequent stimulation of the acinar cell. This latter hypothesis was tested by using atropine (Fig. 1). During perfusion with solutions containing atropine, the enzyme response to excess potassium was abolished. The reduction in volume produced by potassium, however, remained unaffected by atropine. These observations suggest that while the reduction in volume can be attributed to a direct action of potassium on the pancreatic cell, the enzyme secretion may be the result of acetylcholine release from nerve terminals.

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REFERENCES

Bdolah, A., Ben-Zvi, R. & Schramm, M. (1964). *Archs Biochem. Biophys.* **104**, 58–66.

Case, R. M., Harper, A. A. & Scratcherd, T. (1968). *J. Physiol.* **196**, 133–149.

Douglas, W. W. & Poisner, A. M. (1964). *J. Physiol.* **172**, 1–18.

Douglas, W. W. & Rubin, R. P. (1961). *J. Physiol.* **159**, 40–57.

J. Physiol. (1971), **216**, *pp.* 611–624
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STIMULATION OF AMYLASE SECRETION FROM THE PERFUSED CAT PANCREAS BY POTASSIUM AND OTHER ALKALI METAL IONS

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SUMMARY

1. In the isolated cat pancreas, stimulated maximally with secretin, increasing the perfusate potassium concentration (at the expense of sodium ions) caused a copious secretion of amylase from the gland, reduced the volume rate of secretion and caused vasoconstriction.

2. Rubidium and caesium had similar effects to potassium: lithium, though depressing secretory rate, had no effect on enzyme secretion or vasoconstrictor action.

3. Amylase secretion was detected at potassium concentrations of 30 mM and was maximal at 80–90 mM, output declining as the concentration was raised to 120 mM.

4. Amylase secretion was maximal during the first few minutes of exposure to excess potassium, but remained above basal levels throughout the test period. Secretory rate was depressed by a constant amount during the test period.

5. Atropine sulphate blocked the effect on enzyme secretion without affecting the reduction in secretory rate.

6. During perfusion with excess potassium a vasodepressor material with the properties of acetylcholine was detected in the effluent from the gland.

7. The reduction in secretory rate, when perfusate sodium was replaced by potassium, was equal to that obtained when sodium was replaced by sucrose.

8. It is concluded that potassium stimulates amylase secretion indirectly

* Part of this work was carried out during tenure of a Luccock Research Studentship, University of Newcastle upon Tyne.

by releasing acetylcholine from nerve terminals in the gland, and that the reduction in secretory rate is due not to excess potassium but to sodium deficiency.

INTRODUCTION

During a study of the effects of alterations in the ionic environment on water and electrolyte secretion from the perfused cat pancreas (R. M. Case & T. Scratcherd, 1971, in preparation), it became apparent that high concentrations of potassium evoked enzyme secretion from the gland. Potassium is also known to stimulate secretion from several endocrine glands (see Discussion) and to evoke amylase secretion from parotid gland slices (Bdolah, Ben-Zvi & Schramm, 1964).

We have investigated the action of potassium and other alkali metal ions on the secretion of amylase by the saline-perfused pancreas. The results suggest that unlike its action on the adrenal medulla and some other glands (for references see Douglas, 1968), the stimulatory action of potassium on enzyme secretion is not due to a direct effect on the pancreatic acinar cell.

A preliminary report of part of this study has been published (Argent, Case, Poole & Scratcherd, 1970).

METHODS

A saline-perfused preparation of the cat's pancreas (Case, Harper & Scratcherd, 1968) was used in all experiments. Cats of either sex weighing 1·5–2·3 kg were denied food for 18 hr before the experiment. Anaesthesia was induced and maintained by Nembutal (sodium pentobarbitone; 60 mg/kg) administered intraperitoneally.

After the pancreas had been surgically isolated, perfusion fluid was led from a reservoir through a heat exchange coil and by means of a roller pump infused into the gland's arterial supply (the coeliac and superior mesenteric arteries) via a cannula in the aorta. The effluent from the gland was drained through the superior mesenteric vein after occlusion of the portal tract. The standard perfusion fluid, isosmolal with cat's plasma, had the following composition in mM: NaCl 125, KCl 4·3, $NaHCO_3$ 25, $MgCl_2$ 1·0, NaH_2PO_4 1·0, $CaCl_2$ 2·5 and glucose 5. In most experiments choline chloride was added to the perfusion fluids at a concentration of $1{\cdot}5 \times 10^{-3}$ g/l. (Collier, 1969). When either rubidium, caesium, lithium or extra potassium was added to the perfusion fluid (as the chlorides) isosmolality was maintained by omission of appropriate amounts of sodium chloride. The fluids were filtered through Whatman No. 2 paper and gassed continuously with oxygen (95 %) and carbon dioxide (5 %) before use. A bank of four reservoirs allowed rapid changes in the composition of the perfusion fluid to be made.

Throughout all experiments the pancreas was stimulated maximally by infusing secretin (prepared by the method of Crick, Harper & Raper, 1949) into the arterial cannula using a motor-driven syringe. Pancreatic juice samples were weighed and their amylase content estimated by the Nørby method (Lagerlöf, 1942). Amylase is expressed as the total output in international units. The osmolalities of the

perfusion fluids were determined on the Fiske Osmometer (Model G-62, Fiske Associates Inc.) and their sodium and potassium concentrations measured by flame photometry (Mark II, Evans Electroselinium Ltd.).

In some experiments the effluent from the pancreas was assayed for the presence of acetylcholine. The arterial blood pressure of the eserinized eviscerated cat was used for this assay because in this method the interference by potassium is least and in a direction opposite to the effect of acetylcholine (see Brown & Feldberg, 1936). In addition, since isolation of the pancreas incidentally results in an enviscerated cat preparation, it was possible to perform both perfusion and assay experiments concurrently in the same animal. In these experiments therefore the aorta was not cannulated. Instead, the superior mesenteric artery was ligated and the perfusion fluid led directly into the coeliac axis. This procedure had no detectable effect on the functioning of the gland. In all assay experiments, although the circulation through the perfused pancreas and the remainder of the animal appeared not to mix, the coeliac ganglia were ablated to eliminate the possibility of acetylcholine being released from these structures. Samples of effluent were injected into the cat's circulation via a polyethylene catheter in either the external jugular or saphenous vein. To prevent hydrolysis of acetylcholine, eserine sulphate was added both to the perfusion fluids (10 mg/l.) and to the cat's circulation (0·15 mg/kg). Mean arterial blood pressure was measured by means of a Kistler type 412 transducer connected to a polyethylene catheter in the carotid artery and recorded after amplification (Kistler Charge Amplifier Type 566) on a Servoscribe potentiometric recorder (Type RE 511.20).

RESULTS

The effect of potassium ions on pancreatic secretion. In thirty-six experiments, perfusion of the isolated cat pancreas with solutions containing potassium at concentrations of 30–120 mM for periods of 5–80 min stimulated amylase secretion (Fig. 1). The amylase output was greatest during the first few minutes of exposure to excess potassium and remained above basal levels throughout the test period.

Potassium also caused a reduction in the rate of perfusate flow through the gland presumably due to vasoconstriction. In two experiments the -blocking agent phenoxybenzamine (10 mg/l.) lessened this reduction in low rate by a mean of 54 %.

Accompanying the enzyme secretion potassium caused a reduction in the volume of pancreatic secretion (Fig. 1), which remained depressed until a return to normal perfusion fluid was made when the rate of pancreatic secretion was often elevated compared with the control period.

Since an increase in perfusate potassium concentration was always accompanied by an equivalent decrease in sodium concentration, the effect of replacing sodium chloride with isosmotic amounts of sucrose was investigated. This procedure is known to decrease the volume of pancreatic secretion (Case *et al.* 1968). The decrease in secretory rate was linearly related to the decrease in sodium concentration, and was not significantly different when potassium chloride was used instead of sucrose (Fig. 2).

However, replacing sodium chloride with sucrose did not stimulate enzyme secretion nor cause vasoconstriction.

The stimulation of enzyme secretion in relation to potassium concentration. The enzyme stimulating effect of potassium was examined in detail in five experiments. In each experiment four solutions containing different concentrations of potassium were perfused in random order, through the gland for the first 5 min of a 10 min collection period. The amylase output during each test period was expressed as a percentage of the response to a

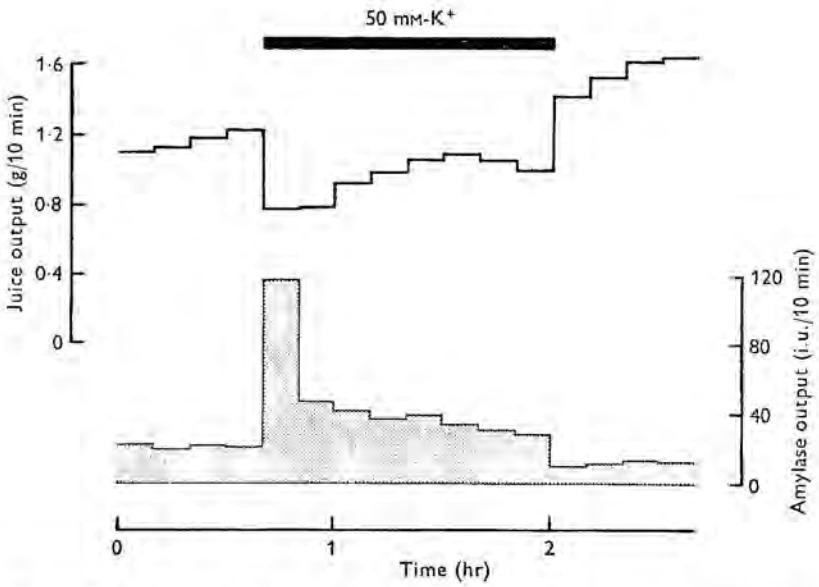

Fig. 1. The effect of replacing extracellular sodium with potassium on the secretory volume and amylase output from an isolated perfused cat pancreas. The gland was stimulated maximally throughout using secretin. For the duration of the horizontal bar the normal perfusate was replaced by one containing 50 mM potassium.

standard dose of acetylcholine (5 μg) given at the end of the experiment. In each experiment the first response was ignored because it was always greater than subsequent stimulations as shown in control experiments where the same potassium concentration was tested repeatedly. The observations from all experiments are combined in Fig. 3 which also illustrates observations from three further experiments in which a total of five different potassium concentrations were tested in a similar way. No enzyme secretion occurred with potassium concentrations below 30 mM. The maximal output of enzyme was observed at potassium concentrations of 80–90 mM. Above this concentration output tended to decline and at

120 mM (the highest concentration tested) the response was not significantly different from that at 50–60 mM.

The effect of atropine on amylase secretion stimulated by potassium. In order to test whether the stimulation of amylase secretion by potassium-rich solutions was due to the direct action of potassium on the acinar cell

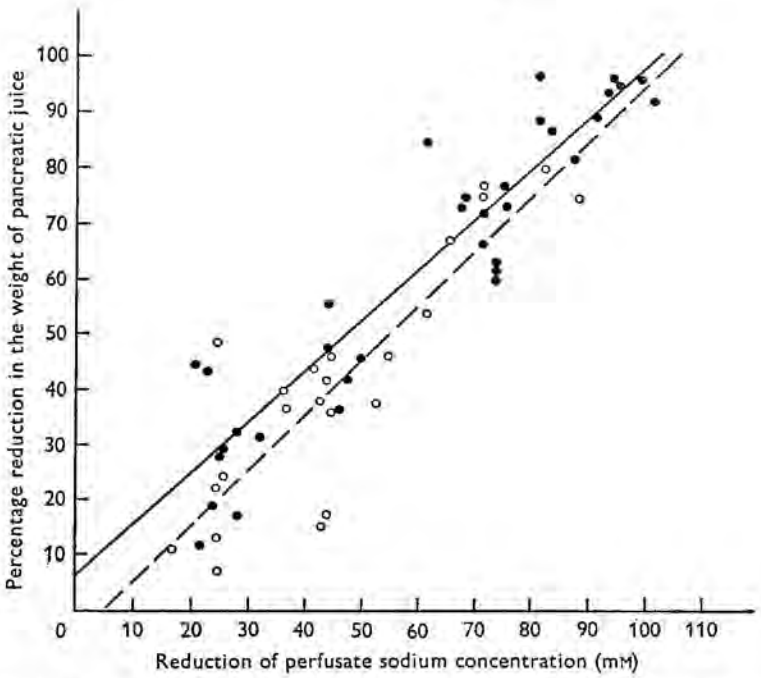

Fig. 2. The effect of reducing the perfusate sodium concentration on the volume of pancreatic juice secreted by the isolated cat pancreas. The open circles represent twenty-three observations in fifteen experiments in which perfusate sodium chloride was replaced by potassium chloride; the interrupted line is the calculated regression line of these observations. The filled circles represent thirty-five observations in ten experiments from an earlier study (Case *et al.* 1968) where sodium chloride was replaced by an osmotically equivalent amount of sucrose; the continuous line is the calculated regression line of these observations. Each point was obtained by expressing the response at equilibrium to the test solution as a percentage of that to normal perfusate. The regression lines which are both statistically significant ($P < 0{\cdot}05$) do not differ from each other ($P < 0{\cdot}05$).

or to the stimulation of cholinergic nerves in the gland, atropine was used. In eleven experiments the addition of atropine sulphate ($7{\cdot}0 \times 10^{-8}$–$1{\cdot}4 \times 10^{-5}$ M) abolished the enzyme secretion in response to potassium, though the reduction in pancreatic juice volume persisted. Fig. 4 illustrates an experiment in which the isolated pancreas was perfused with a potas-

sium-rich solution (50 mM) which stimulated a copious secretion of amylase. Atropine ($1{\cdot}4 \times 10^{-7}$ M) abolished the effect on enzyme secretion. One hour after cessation of atropine infusion potassium (50 mM) again stimulated the secretion of amylase. Atropine itself had no effect on the basal secretion of enzymes.

The presence of a vasodepressor material (acetylcholine) in the effluent from the gland. The inhibition of enzyme secretion by atropine suggested that potassium acts on cholinergic nerves. Further evidence that acetylcholine

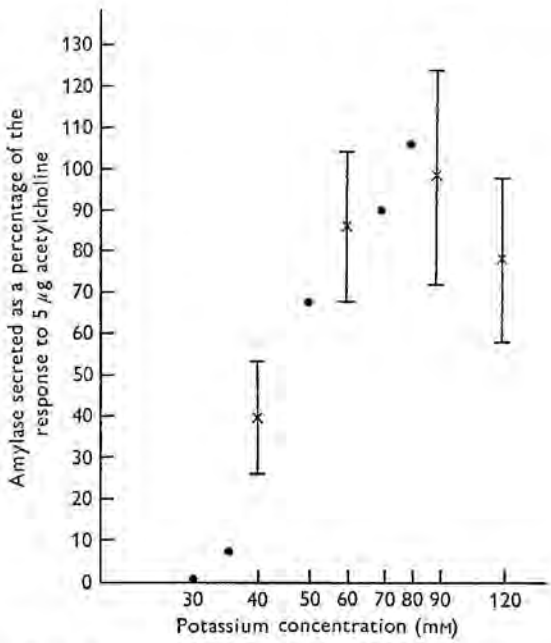

Fig. 3. The effect of potassium-rich perfusates on amylase secretion from the isolated cat pancreas The total amount of amylase secreted in response to a 5 min perfusion with a given potassium concentration is expressed as a percentage of the response to 5 μg acetylcholine chloride. The order in which the various concentrations of potassium were tested in different experiments was random. × = mean (± S.E. of mean) of five observations from five experiments. ● = mean of two observations from a total of three experiments.

is the mediator of the response was obtained from analysis of the effluent from the gland. In seven experiments samples of effluent were collected before, during and after perfusing the gland with a potassium-rich solution. Aliquots of each sample (0·5 or 1·0 ml.) were injected into the cat's jugular or saphenous vein and changes in blood pressure monitored. All experiments gave similar results to those illustrated in Fig. 5, which is

from the same experiment as Fig. 4. The injection of effluent collected during a 5 min perfusion with 50 mM potassium caused a fall of blood pressure, approximately equal to that obtained by injecting 20 ng acetylcholine. Vasodepressor material was also present in effluent collected during the 5 min period immediately after perfusion with 50 mM potassium but not in the subsequent collection (Fig. 5*A*). Similar observations were made after atropine ($1{\cdot}4 \times 10^{-7}$ M) has been added to the perfusion fluid (Fig. 5*B*) although atropine abolished the amylase secretion in

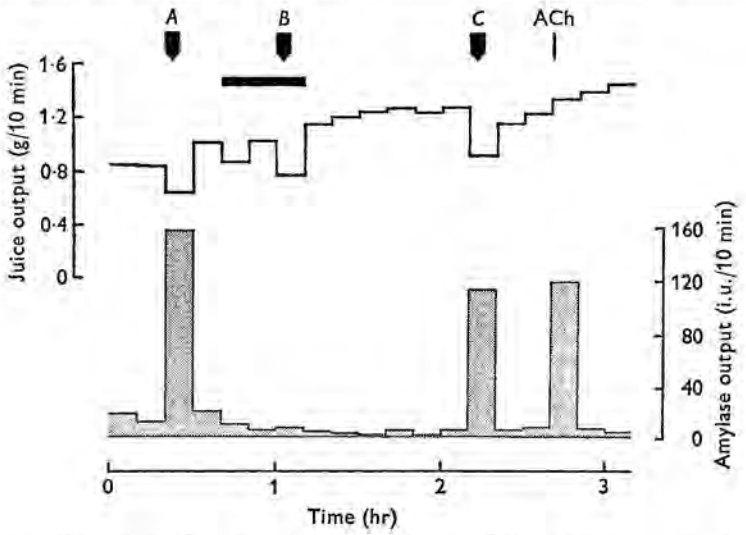

Fig. 4. The effect of atropine on the volume and amylase responses of the perfused cat pancreas to excess potassium. The gland was stimulated maximally throughout using secretin. The perfusion fluids contained atropine sulphate ($1{\cdot}4 \times 10^{-7}$ M) for the length of the horizontal bar. The broad arrows, lettered *A*, *B* and *C*, indicate 5 min periods during which the perfusate contained 50 mM potassium. The arrow marked ACh indicates a single injection of 5 μg acetylcholine chloride.

response to potassium (Fig. 4). During the third period of stimulation with 50 mM potassium, after atropine had been removed from the perfusion fluid, the vasodepressor material was again detected in the effluent from the gland (Fig. 5*C*).

Effluent of normal composition had no effect on blood pressure. Potassium-rich perfusion fluid (i.e. before entering the gland) caused a slight rise in blood pressure. The depressor effect of active effluents was abolished either by making them alkaline with sodium hydroxide, or by atropinizing the cat (0·8 mg/kg). After these procedures the effluents had a pressor effect slightly greater than that obtained with potassium-rich perfusion fluid.

These observations suggest that acetylcholine is the depressor substance in the effluent, and is presumably released from nerve terminals in the gland by the high potassium concentrations. The pressor responses observed after blocking the depressor activity suggests that potassium may also release small amounts of catecholamines from sympathetic nerve terminals within the gland.

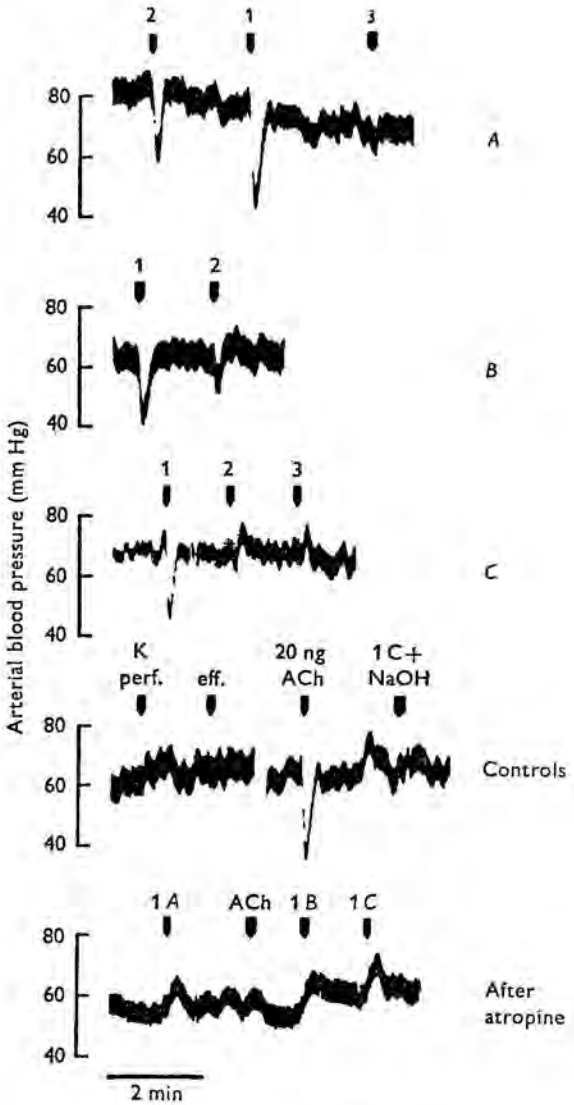

Fig. 5. For legend see opposite page.

The stimulatory effect on amylase secretion of other alkali metal ions. Several experiments were performed to ascertain whether amylase secretion could be evoked by alkali metal ions other than potassium.

In three experiments lithium at concentrations of 30–120 mM did not stimulate the secretion of enzymes from the pancreas. Rubidium and caesium however had effects similar to potassium. In four experiments the effects of rubidium and caesium were compared at two concentrations, 40 and 90 mM (Table 1). The design of the experiments was similar to that used in testing different concentrations of potassium, the response to each

TABLE 1. Comparison of the effectiveness of rubidium, caesium and potassium at 40 mM and 90 mM concentrations in stimulating amylase secretion from isolated cat pancreas (four experiments)

	40 mM			90 mM		
	Rb	Cs	K	Rb	Cs	K
Mean	64·75	113·3	39	71·75	174·3	97·4
± S.E. of mean	±15·48	±17·11	±13·5	±19·72	±38·3	±26·26

Amylase secreted is expressed as a % of the response to 5 μg acetylcholine.

test fluid (given in random order) being compared with the response to 5 μg acetylcholine (given last). At both concentrations the mean amylase output in response to caesium was greater than that in response to rubidium and potassium although only the difference between potassium and caesium at 40 mM was statistically significant ($P < 0{\cdot}02$). The response to

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Legend to Fig. 5.

Fig. 5. The detection of acetylcholine in the effluent from the perfused pancreas. Acetylcholine was assayed by its depressor action on the arterial blood pressure of the same animal in which the perfusion experiment was performed (see Methods). The effluents tested were those collected in the experiment illustrated in Fig. 4. During each 5 min perfusion with 50 mM potassium (lettered *A*, *B* and *C*; see Fig. 4) the total volume of effluent was collected and labelled sample 1. The effluent collected in the two succeeding 5 min periods (during perfusion with normal perfusate) was labelled sample 2 and 3 respectively. The effect of 1·0 ml. aliquots of all these samples is shown in the upper three blood pressure traces. A series of controls shows the effect of injecting potassium-rich perfusate (K perf.), effluent of normal composition (eff.), a 20 ng dose of acetylcholine chloride (20 ng ACh) and a sample of potassium-rich effluent after treatment with sodium hydroxide (1C+NaOH). The lowest trace illustrates the effect of potassium-rich effluents and acetylcholine chloride (20 ng) after the cat had been given atropine sulphate (0·8 mg/kg).

rubidium and potassium did not differ significantly at either concentration ($P > 0{\cdot}1$).

Lithium, rubidium and caesium all reduced the volume of pancreatic secretion but only rubidium and caesium significantly reduced the rate of perfusate flow through the gland. The reduction in perfusate flow observed with caesium was usually less than that observed with either potassium or rubidium.

DISCUSSION

The action of potassium in releasing amylase from the pancreatic acinar cell was not unexpected as many precedents exist for the liberation of both hormones and transmitter substances by potassium. The effects of rapid intra-arterial injections of potassium chloride in causing the secretion of adrenaline from the adrenal medulla were reviewed as early as 1940 by Fenn. Since then the number of hormones shown to be released by high potassium concentration in the extracellular fluid has steadily increased: vasopressin (Douglas & Poisner, 1964), thyrotrophin and adrenocorticotrophic hormone (Vale & Guillemin, 1967), luteinizing hormone (Samli & Geschwind, 1968), follicle stimulating hormone (Jutisz & Paloma de la Llosa, 1970) and growth hormone (MacLeod & Fontham, 1970). Elevated potassium concentration also stimulates the release of insulin from both perfused (Grodsky & Bennett, 1966) and cultured (Lambert, Jeanrenaud, Junod & Renold, 1969) rat pancreas and from rabbit pancreas *in vitro* (Hales & Milner, 1968). Potassium ions also have a stimulatory action on exocrine glands. Bdolah *et al.* (1964) have reported that amylase secretion is stimulated from rat parotid gland slices when the concentration of potassium is raised to 20–60 mM, conditions which also cause an increase in the concentration of cyclic 3′,5′-adenosine monophosphate in this tissue (Rasmussen & Tenenhouse, 1968).

The results presented in this paper demonstrate that perfusing the isolated cat pancreas with potassium-rich solutions stimulates the secretion of amylase. Quantitatively the 'dose–response' curve for potassium-stimulated amylase secretion (Fig. 3) is similar to that obtained by Douglas & Poisner (1964) for the release of vasopressin from the rat neurohypophysis. The effect of atropine and the detection of acetylcholine in the effluent from the gland, suggest that the stimulatory effect of potassium is an indirect one secondary to the release of acetylcholine.

As early as 1936 Brown & Feldberg demonstrated that potassium and to a lesser extent caesium would release acetylcholine from the perfused superior cervical ganglion of the cat. Furthermore, Feldberg & Guimarãis (1936) observed the release of acetylcholine by potassium, but not caesium, from the submaxillary gland of both cat and dog and from the sweat glands

and tongue of the cat. Acetylcholine also appeared in the effluent from the perfused frog heart when Ringer solution containing excess potassium was used (Beznák, 1934). Confirmation of the action of potassium on nervous tissue came when Mann, Tennenbaum & Quastel (1939) demonstrated that potassium, rubidium and to a lesser extent caesium released acetylcholine from respiring brain slices. Our finding that caesium was more effective in stimulating amylase secretion from the isolated pancreas than either potassium or rubidium is difficult to reconcile with these early papers in which caesium was usually less effective in liberating acetylcholine.

Acetylcholine has been assumed to be the transmitter substance responsible for enzyme secretion by the pancreas as a result of anatomical studies (Coupland, 1958) and the effects of atropine (Brown, Harper & Scratcherd, 1967) but it has not been identified in the blood or perfusate issuing from the pancreas after vagal stimulation. The action of potassium reported in this paper seems to be due to release of acetylcholine from vagal post-ganglionic nerve terminals, thus providing further evidence for acetylcholine being the transmitter substance. However, potassium would be expected to stimulate preganglionic nerve terminals as well as post-ganglionic nerves. Evidence that the liberation of amylase depends on the action of potassium on post-ganglionic nerves was obtained by using hexamethonium to block ganglionic transmission. In the intact cat hexamethonium blocks the secretion of amylase in response to vagal stimulation (Brown *et al.* 1967) whereas the addition of hexamethonium bromide (10 mg/l.) to the perfusate of the isolated gland had no effect on the amylase response to potassium.

Since potassium indirectly stimulates the secretion of amylase from the perfused cat pancreas the question must be raised as to whether potassium has an indirect action on other secretory tissue. The observation that potassium has a direct effect on the chromaffin cells of the adrenal medulla (Vogt, 1952; Douglas & Rubin, 1961) may also apply to the *in vitro* rat neurohypophysis since neither acetylcholine, carbacol nor eserine stimulate the release of vasopressin (Douglas & Poisner, 1964). However, Schramm (1968) has shown that potassium ions stimulate amylase secretion from rat parotid gland indirectly, by liberating stores of endogenous catecholamines. The reported effects of potassium on insulin secretion may be secondary to the release of acetylcholine since stimulation of the vagal supply to the perfused rabbit pancreas increases the rate of insulin secretion from the gland (Findlay, Gill, Lever, Randle & Spriggs, 1969). Also in isolated rat pancreatic tissue acetylcholine potentiates insulin secretion stimulated by glucose (Malaisse, Malaisse-Lagae, Wright & Ashmore, 1967). None of the studies on hormone release from the adenohypophysis have included the effects of neurotransmitter substances and

blocking agents. It may be that secretory tissue of neural origin, viz. nerve terminals, sympathetic ganglia, the neurohypophysis and adrenal medulla, are all directly stimulated by potassium whereas other secretory tissues are not.

Besides stimulating amylase secretion excess potassium decreased both the rate of perfusion and the volume of pancreatic juice secreted in response to secretin. The fall in perfusion rate was presumably due to vasoconstriction as was observed by Brown & Feldberg (1936) in the perfused superior cervical ganglia. In our experiments the vasoconstriction was probably partly due to the liberation of catecholamines from sympathetic nerve terminals since it was substantially decreased after α-receptor blockade. Furthermore, the potassium-rich effluent contained more pressor activity than could be accounted for by its potassium content when tested on the atropinized eviscerated cat. The decrease in secretion rate was not secondary to the decreased perfusion rate since decreasing the flow of normal perfusate to levels observed during potassium stimulation had little or no effect on secretory rate. Similarly, when lithium was tested in place of potassium the reduction in secretory rate persisted in the absence of any effect on perfusate flow rate. It seems more likely that the reduction in secretory rate can be explained by the decrease in sodium concentration of the perfusion fluid since it is known that the volume of pancreatic secretion is reduced under these conditions (Case *et al.* 1968). If sodium lack alone is responsible for the reduction in secretory rate the effect of replacing sodium by potassium ions should be approximately equal to that obtained using sucrose. This was found to be so (Fig. 2).

In summary, replacement of perfusate sodium by potassium has three effects on the saline perfused cat pancreas. First, it causes a reduction in perfusion rate, by the action of potassium on the vasculature of the gland and possibly also indirectly due to release of catecholamines; secondly it causes a reduction in secretory rate due to the reduction in sodium concentration, and thirdly it causes copious amylase secretion because potassium releases acetylcholine from nerve terminals in the gland. This latter indirect action of potassium is similar to that in the parotid gland (Schramm, 1968) but different from that in the adrenal medulla and neurohypophysis (Douglas, 1968).

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REFERENCES

ARGENT, B. E., CASE, R. M., POOLE, L. M. & SCRATCHERD, T. (1970). Stimulation of enzyme secretion from the perfused cat pancreas by potassium. *J. Physiol.* **208**, 79–80*P*.

BDOLAH, A., BEN-ZVI, R. & SCHRAMM, M. (1964). The mechanism of enzyme secretion by the cell. II. Secretion of amylase and other proteins by slices of rat parotid gland. *Archs Biochem. Biophys.* **104**, 58–66.

BEZNÁK, A. B. L. (1934). On the mechanism of the autacoid function of parasympathetic nerves. *J. Physiol.* **82**, 129–153.

BROWN, G. L. & FELDBERG, W. (1936). The action of potassium on the superior cervical ganglion of the cat. *J. Physiol.* **86**, 290–305.

BROWN, J. C., HARPER, A. A. & SCRATCHERD, T. (1967). Potentiation of secretin stimulation of the pancreas. *J. Physiol.* **190**, 519–530.

CASE, R. M., HARPER, A. A. & SCRATCHERD, T. (1968). Water and electrolyte secretion by the perfused pancreas of the cat. *J. Physiol.* **196**, 133–149.

COLLIER, B. (1969). The preferential release of newly synthesized transmitter by a sympathetic ganglion. *J. Physiol.* **205**, 341–352.

COUPLAND, R. E. (1958). The innervation of pancreas of the rat, cat and rabbit as revealed by the cholinesterase technique. *J. Anat.* **92**, 143–149.

CRICK, J., HARPER, A. A. & RAPER, H. S. (1949). On the preparation of secretin and pancreozymin. *J. Physiol.* **110**, 367–376.

DOUGLAS, W. W. (1968). Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmac.* **34**, 451–474.

DOUGLAS, W. W. & POISNER, A. M. (1964). Stimulus-secretion coupling in a neurosecretory organ: the role of calcium in the release of vasopressin from the neurohypophysis. *J. Physiol.* **172**, 1–18.

DOUGLAS, W. W. & RUBIN, R. P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol.* **159**, 40–57.

FELDBERG, W. & GUIMARÃIS, J. A. (1936). The liberation of acetylcholine by potassium. *J. Physiol.* **86**, 306–314.

FENN, W. O. (1940). The role of potassium in physiological processes. *Physiol. Rev.* **20**, 377–415.

FINDLAY, J. A., GILL, J. R., LEVER, J. D., RANDLE, P. J. & SPRIGGS, T. L. B. (1969). Increased insulin output following stimulation of the vagal supply to the perfused rabbit pancreas. *J. Anat.* **104**, 580.

GRODSKY, G. M. & BENNETT, L. L. (1966). Cation requirements for insulin secretion in the isolated perfused pancreas. *Diabetes* **15**, 910–913.

HALES, C. N. & MILNER, R. D. G. (1968). The role of sodium and potassium in insulin secretion from rabbit pancreas. *J. Physiol.* **194**, 725–743.

JUTISZ, M. & PALOMA DE LA LLOSA, M. (1970). Requirement of Ca^{++} and Mg^{++} ions for the *in vitro* release of follicle-stimulating hormone from rat pituitary glands and in its subsequent biosynthesis. *Endocrinology* **86**, 761–768.

LAGERLÖF, H. O. (1942). *Pancreatic Function and Pancreatic Disease Studied by Means of Secretin.* Stockholm: Norstedt & Söner.

LAMBERT, A. E., JENRENAUD, B., JUNOD, A. & RENOLD, A. E. (1969). Organ culture of fetal rat pancreas. II. Insulin release induced by amino and organic acids, by hormonal peptides, by cationic alterations of the medium and by other agents. *Biochim. biophys. Acta* **184**, 540–553.

MACLEOD, R. M. & FONTHAM, E. H. (1970). Influence of ionic environment on the *in vitro* synthesis and release of pituitary hormones. *Endocrinology* **86**, 863–869.

MALAISSE, W., MALAISSE-LAGAE, F., WRIGHT, P. H. & ASHMORE, J. (1967). Effects of adrenergic and cholinergic agents upon insulin secretion *in vitro*. *Endocrinology* **80**, 975–978.

MANN, P. J. G., TENNENBAUM, M. & QUASTEL, J. H. (1939). Acetylcholine metabolism in the central nervous system. The effects of potassium and other cations on acetylcholine liberation. *Biochem. J.* **33**, 822–835.

RASMUSSEN, H. & TENENHOUSE, A. (1968). Cyclic adenosine monophosphate, Ca^{++}, and membranes. *Proc. natn. Acad. Sci. U.S.A.* **59**, 1364–1370.

SAMLI, M. H. & GESCHWIND, I. I. (1968). Some effects of energy-transfer inhibitors and of Ca^{++}-free or K^{+}-enhanced media on the release of luteinizing hormone (LH) from the rat pituitary gland *in vitro*. *Endocrinology* **82**, 225–231.

SCHRAMM, M. (1968). Amlyase secretion in rat parotid slices by apparent activation of endogenous catecholamine. *Biochim. biophys. Acta* **165**, 546–549.

VALE, W. & GUILLEMIN, R. (1967). Potassium-induced stimulation of thyrotropin release *invitro*. Requirement for presence of calcium and inhibition by thyroxine. *Experientia* **23**, 855–857.

VOGT, M. (1952). The secretion of the denervated adrenal medulla of the cat. *Br. J. Pharmac.* **7**, 325–330.

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The effects of calcium on volume and amylase secretion from the perfused cat pancreas

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There is evidence to suggest that calcium ions play an important role in the secretory mechanisms of various cell types (Rubin, 1970). We have tested the effect of alterations in the extracellular calcium concentration on the volume and amylase secretion from a perfused preparation of the cat pancreas (Case, Harper & Scratcherd, 1968).

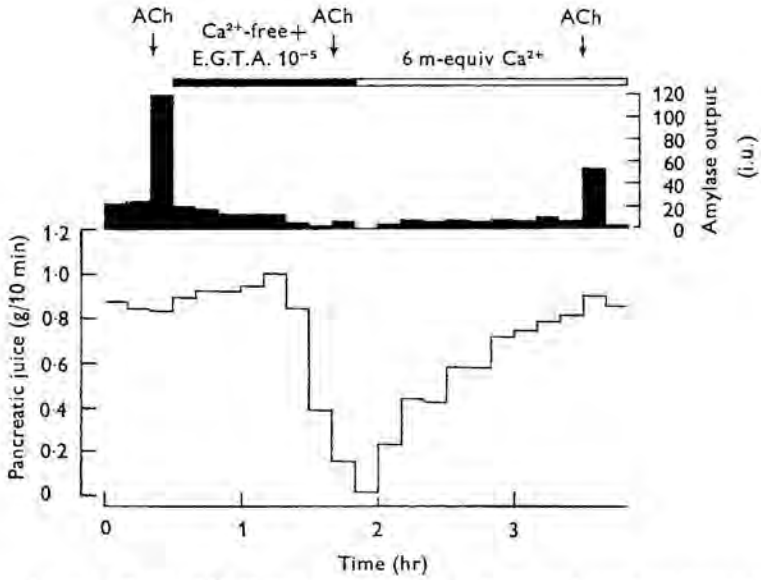

Fig. 1. The effects of Ca^{2+}-free perfusate on volume and amylase secretion from the isolated cat pancreas. The gland was maximally stimulated throughout by infusing secretin at a supramaximal dose (30 μg/min). The calcium concentration of the perfusion fluid varied from normal (2·5 m-equiv Ca^{2+}/l.) for the periods indicated by the length of the horizontal bars. The arrows indicate single injections of 1 μg acetylcholine.

Ca^{2+}-free perfusates containing the specific calcium chelator EGTA (ethyleneglycol-bis-(β-amino ethyl ether)-*N*,*N*′-tetraacetic acid) (10^{-5} M) initially caused a small reduction in the amount of amylase secreted in response to single doses of acetylcholine, but had no effect on the volume

[P.T.O.

of pancreatic secretion. Prolonging the exposure to Ca^{2+}-free solutions caused a progressive reduction in the secretory rate (Fig. 1). Acetylcholine administered during the latter part of this phase of reduced secretory rate did not stimulate amylase secretion (Fig. 1). Similar results were obtained when pancreozymin was used as the enzyme stimulant. Increasing the perfusate EGTA concentration decreased the time for the reduction in secretory rate to become apparent. The effects of Ca^{2+}-free solutions were only partially reversible by normal perfusate (2·5 m-equiv Ca^{2+}/l.) but completely reversed by Ca^{2+}-rich perfusate (6–10 m-equiv Ca^{2+}/l.) (Fig.1).

Ca^{2+}-rich perfusate (10 m-equiv Ca^{2+}/l.) had no effect on the rate of pancreatic secretion when the gland was secreting either maximally or submaximally in response to secretin. However, if the gland was stimulated to secrete amylase at a submaximal rate by the slow infusion of acetylcholine or pancreozymin an increase in perfusate calcium concentration resulted in a large increase in amylase secretion.

These results thus confirm the importance of calcium ions in pancreatic enzyme secretion, first demonstrated by Hokin (1966), and extend this observation to include water and electrolyte secretion.

REFERENCES

Case, R. M., Harper, A. A. & Scratcherd, T. (1968). *J. Physiol.* **196**, 133–149.
Hokin, L. E. (1966). *Biochim. biophys. Acta* **115**, 219–221.
Rubin, R. P. (1970). *Pharmac. Rev.* **22**, 389–428.

J. Physiol. (1973), **230**, *pp.* 575–593
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AMYLASE SECRETION BY THE PERFUSED CAT PANCREAS IN RELATION TO THE SECRETION OF CALCIUM AND OTHER ELECTROLYTES AND AS INFLUENCED BY THE EXTERNAL IONIC ENVIRONMENT*

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SUMMARY

1. Amylase secretion from the perfused pancreas consists of two components: a small continuous basal secretion and a stimulated secretion in response to acetylcholine or cholecystokinin-pancreozymin. The response to small doses of either stimulant was repeatable over several hours.

2. The calcium concentration of pancreatic juice, always less than that of the perfusate, was normally constant above secretory rates of 0·15 g/10 min. However, when the concentration of enzymes in the juice rose, either after stimulation or at very low secretory rates, the calcium concentration rose in parallel, suggesting that this calcium is bound to, or is a component of, pancreatic enzymes.

3. Elevation of the perfusate calcium concentration resulted in a parallel increase in the calcium concentration of the pancreatic juice.

4. Calcium-free solutions initially caused a small reduction in basal and stimulated amylase secretion and, after prolonged periods of perfusion, abolished stimulated secretion and caused a reduction in electrolyte secretion. The latter was completely reversed by calcium-rich perfusates but the effects on enzyme secretion were only partially reversible.

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5. Calcium-rich perfusates had no effect on the rate of electrolyte secretion but potentiated submaximally stimulated amylase secretion.

6. Barium did not substitute for calcium in supporting pancreatic secretion.

7. Alterations in the extracellular concentrations of sodium, potassium and magnesium had no *direct* effect on amylase secretion.

8. The local anaesthetic tetracaine inhibited amylase secretion at a lower concentration than that required to inhibit electrolyte secretion.

9. It is concluded (*a*) that calcium is secreted into the pancreatic juice in two fractions, one associated with enzymes and the other with the electrolyte component of the juice; and (*b*) that calcium ions play an important role in the stimulus-secretion coupling of pancreatic acinar cells, but that the effects of calcium depletion on electrolyte secretion may principally be due to alterations in the permeability of the duct system.

INTRODUCTION

The essential features of the synthesis, intracellular transport, and storage of digestive enzymes within the pancreatic acinar cell are well defined. After synthesis on the ribosomes of the rough-surfaced endoplasmic reticulum, the digestive enzymes, or their zymogens, are transferred via the cisternae of the rough-surfaced endoplasmic reticulum and the small vesicles of the peripheral Golgi complex to the condensing vacuoles, which are subsequently transformed into zymogen granules by progressive filling and concentration of their contents and stored in the apical region of the cell. Morphological studies have shown that, following stimulation, zymogen discharge (which we shall refer to as secretion) involves the movement of the granule to the cell surface, where its membrane fuses with the plasma membrane thus extruding its contents by exocytosis into the acinar lumen (for review see Schramm, 1967). However, little is known of the mechanical processes involved in movement of the zymogen granules, or how they are controlled (stimulus-secretion coupling).

This study explores the influence of extracellular ionic composition on these processes in the perfused cat pancreas. Such an analysis in the intact gland is complicated by the need for a background secretion of electrolytes and water (to act as a vehicle for the enzymes), which itself is markedly influenced by the composition of the perfusing fluid (Case, Harper & Scratcherd, 1968, 1969*b*). Nevertheless a perfused preparation does offer considerable advantages over alternative *in vitro* techniques, notably rapid and reversible alteration of the extracellular fluid composition, efficient oxygenation and collection of uncontaminated secretory products. In addition, the simultaneous measurement of electrolyte secretion has

allowed observations to be made on this component of the pancreatic juice.

The present observations (previously published in brief; Argent, Case, Fraser & Scratcherd, 1972) suggest that in the pancreas, as in some other secretory tissues (see, Rubin, 1970), the calcium ion plays an important role in enzyme secretion, but that other extracellular cations, magnesium, sodium and potassium, are not directly involved.

METHODS

A saline-perfused preparation of the cat's pancreas (Case *et al.* 1968) was used in all experiments. Cats of either sex, weighing 0·4–4·2 kg and denied food for 18 hr before the experiment, were anaesthetized with Nembutal (sodium pentobarbitone, 60 mg/kg I.P.) and the pancreas surgically isolated. Perfusion fluid was led from a reservoir through a heat-exchange coil and, by means of a roller pump, infused into the gland's arterial supply (the coeliac and superior mesenteric arteries) via a cannula in the aorta. The effluent from the gland was drained through the superior mesenteric vein after occlusion of the portal tract. The standard perfusion fluid isosmolal with cat's plasma had the following composition in mM: NaCl 125, KCl 4·3, $NaHCO_3$ 25, $MgCl_2$ 0·5, NaH_2PO_4 1·0, $CaCl_2$ 1·25 and glucose 5. Where perfusate potassium, magnesium or calcium concentrations were altered, isomolality was maintained by adjusting the sodium ion concentration; when sodium was removed, an osmotically equivalent amount of sucrose was added. The calcium chelator EGTA (ethylene-glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid) was added to calcium-free perfusates at concentrations specified in the text. The fluids were filtered through Whatman no. 2 paper before use and gassed continuously with oxygen (95 %) and carbon dioxide (5 %). A bank of four reservoirs allowed rapid changes in the composition of the perfusion fluids to be made.

In all experiments electrolyte secretion was stimulated by infusing secretin into the arterial cannula using a motor-driven syringe. Enzyme secretion was stimulated by rapid pulses or prolonged infusions of acetylcholine chloride or, less frequently, of cholecystokinin-pancreozymin (CCK-Pz). Secretin and CCK-Pz were prepared by the method of Crick, Harper & Raper (1949), though in some experiments purer preparations (G.I.H. Laboratory, Karolinska Institutet, Stockholm) were used.

Pancreatic juice samples were collected in plastic tubes and weighed. Either or both of two indices of enzyme secretion were measured: total protein (mg), estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), and amylase activity (i.u.), estimated by the Nørby method (Lagerlöf, 1942). The concentrations of calcium and magnesium in pancreatic juice and perfusion fluids were estimated by atomic absorption spectrometry (Unicam SP 90) and sodium and potassium by flame photometry (Mark II, Evans Electroselenium Ltd). The osmolalities of the perfusion fluids were determined on the Osmet Precision Osmometer (Precision Systems Ltd).

Where statistical analysis has been employed results are expressed as the mean ± S.E.

RESULTS

Normal perfusate

Enzyme secretion by the perfused cat pancreas. There was usually no measurable secretion from the unstimulated saline-perfused cat's pancreas. In the absence of an electrolyte secretion, CCK-Pz and acetylcholine were unable to evoke any detectable amylase secretion. During secretin stimulated electrolyte secretion amylase secretion consisted of two components: a small continuous basal secretion and a stimulated secretion

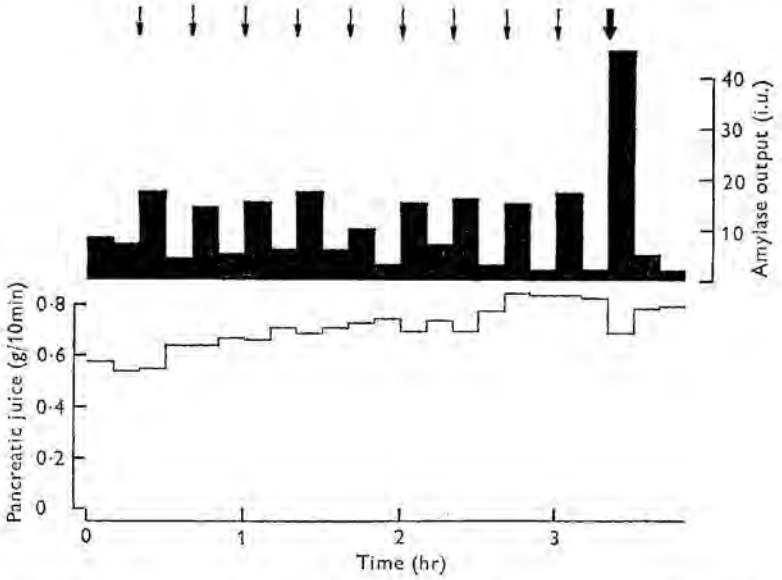

Fig. 1. The secretion of amylase by the perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min). The thin arrows represent single injections of 0·1 mg of CCK-Pz prepared by the method of Crick, Harper & Raper (1949). The thick arrow represents a larger dose (1·0 mg) of the same stimulant.

which was dose-dependent and occurred in response to exogenously administered acetylcholine or CCK-Pz. The rate of basal amylase secretion from the gland varied in different animals. Control experiments demonstrated that a fall in basal amylase secretion normally occurred during the course of an experiment (Fig. 1). If the perfused gland was stimulated to secrete large amounts of enzyme the response to the same dose of stimulant decreased during the course of an experiment. With small doses the secretory response was repeatable over several hours (Fig. 1), though the

response to the first dose of enzyme stimulant was often greater than subsequent responses, and has therefore been ignored in all experiments.

The calcium content of pancreatic juice. The concentration of calcium in pancreatic juice collected from glands stimulated maximally by secretin was always less than that of the perfusion fluid. In eleven experiments in which the gland was perfused with normal perfusate ($2{\cdot}82 \pm 0{\cdot}09$ m-equiv Ca/l.) the mean concentration of calcium in the pancreatic juice was

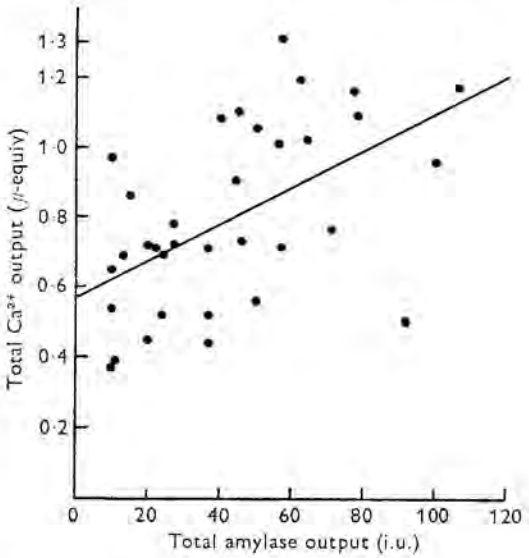

Fig. 2. The secretion of calcium and amylase in pancreatic juice. The points represent a total of thirty-five observations from twelve experiments in which glands were stimulated to secrete amylase by various doses of acetylcholine (25 ng–1 μg). The solid line is a calculated regression line ($P = <0{\cdot}001$).

$0{\cdot}63 \pm 0{\cdot}04$ m-equiv/l. ($n = 78$). At secretory rates greater than 0·15 g/10 min the concentration of calcium was independent of flow rate. In one experiment where the gland was stimulated to secrete at flow rates below 0·15 g/10 min the concentration of calcium in the juice increased with decreasing flow rate, as did the concentration of amylase.

Following stimulation by acetylcholine the output of calcium in the pancreatic juice increased in proportion to the total amount of amylase secreted (Fig. 2). This increased output of calcium paralleled the increased output of amylase when the stimulant was administered as a pulse or as an infusion (Fig. 3).

The effects of tetracaine on pancreatic secretion. In two experiments

perfusing the isolated pancreas with solutions containing the local anaesthetic tetracaine ($2{\cdot}0$–$5{\cdot}0 \times 10^{-4}$ M) reduced the secretion of amylase in response to single doses of acetylcholine but had no effect on the volume of pancreatic secretion (Fig. 4). In three experiments concentrations of tetracaine greater than 10^{-3} M caused a reduction in the volume of pancreatic secretion.

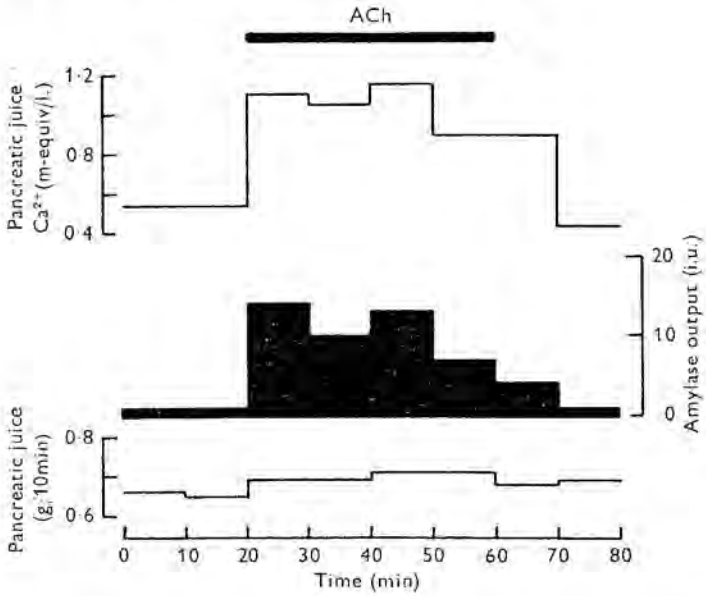

Fig. 3. Parallel output of calcium and amylase during acetylcholine infusion. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min). Acetylcholine (ACh) was infused at a rate of 20 ng/min for the duration of the horizontal bar.

Alterations in perfusate [Ca] concentration

The effects of calcium-free perfusate on electrolyte and enzyme secretion. In nine experiments prolonged perfusion with calcium-free solutions containing EGTA (10^{-5} M) caused a progressive inhibition of electrolyte secretion which became apparent after 50–70 min (Fig. 5). In the early stages this inhibition could be reversed by a return to normal perfusate but when the effect had become marked, perfusion with calcium-rich fluids (6–10 m-equiv Ca/l.) was necessary for complete reversal. By increasing the EGTA concentration of calcium-free perfusates, the time required for inhibition to become apparent was reduced. With a solution containing 10^{-3} M-EGTA, the secretory rate in two experiments was reduced by a mean of 72 % after only 20 min. EGTA itself was not

responsible for the inhibition for, when added to normal (calcium-containing) perfusate, in concentrations up to 10^{-3} M, it had no inhibitory effects (two experiments).

Although reduced, basal enzyme secretion was always detectable during perfusion with calcium-free EGTA solutions, provided electrolyte secretion was maintained (Figs. 5, 6). During either the initial period of calcium-free perfusion (when electrolyte secretion was unaffected) or the early phase of

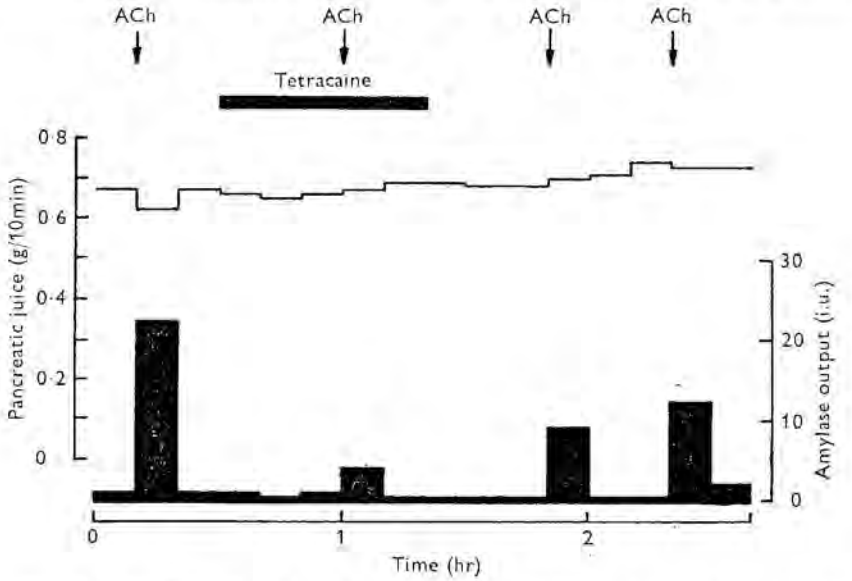

Fig. 4. The effects of the local anaesthetic tetracaine on amylase secretion. For the period denoted by the horizontal bar the fluid perfusing the gland contained tetracaine ($2{\cdot}0 \times 10^{-4}$ M). The arrows marked ACh denote single injections of 200 ng acetylcholine. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min).

reduced secretory rate the response to acetylcholine was slightly diminished (Fig. 6). During the latter stages of reduced electrolyte secretion the response to acetylcholine was virtually abolished (Fig. 5), and, unlike electrolyte secretion, it was not fully restored by perfusion with calcium-rich fluids.

On returning to normal perfusate after prolonged calcium-free perfusion the concentration of calcium in pancreatic juice was elevated when compared to the control period and often equal to the concentration in the perfusing fluid (Fig. 5).

Calcium-free solutions had no effect on the rate of perfusion through the gland. In this series of experiments, similar results were obtained when

CCK-Pz and pure secretin were substituted for acetylcholine and crude secretin respectively.

The effects of calcium-rich perfusates on pancreatic secretion. Perfusion with fluids containing 10 m-equiv Ca/l. did not affect the rate of pancreatic secretion stimulated maximally or submaximally by secretin, but did cause the juice calcium concentration to rise and remain elevated throughout the 30 min test period (Fig. 7). In three of seven such experiments a small increase (mean 120 %) in basal amylase secretion was also observed on switching to calcium-rich buffer, but the effect was transient, being observed only in the first 10 min period of perfusion.

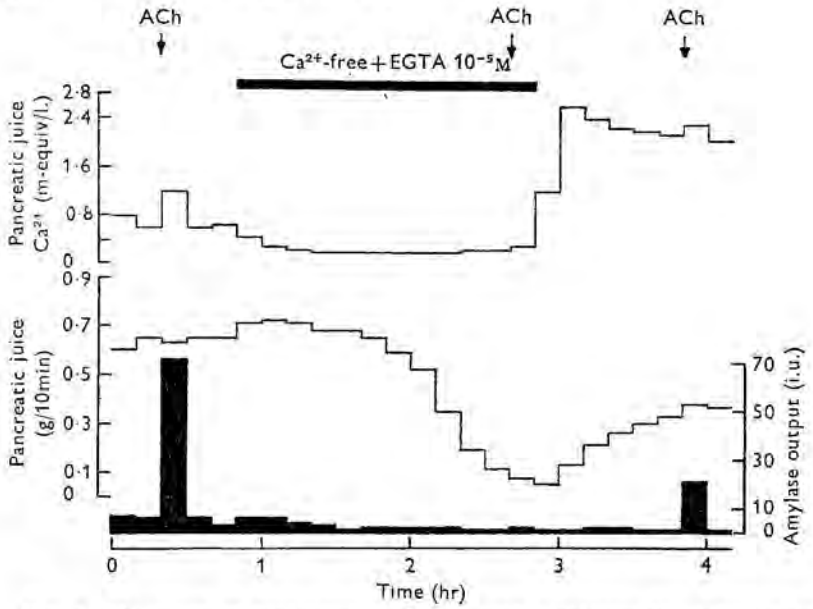

Fig. 5. The effect of prolonged perfusion with a calcium-free solution on electrolyte and amylase secretion from a perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min). A Ca-free solution containing EGTA (10^{-5} M) was perfused through the gland for the duration of the horizontal bar. The arrows marked ACh indicate single injections of acetylcholine (ACh, 200 ng).

In three further experiments, increasing the calcium concentration of the perfusate during minimal acetylcholine infusion (5 ng/min) caused a large increase in amylase secretion (Fig. 8). This effect was not wholly due to the release of acetylcholine from nerve terminals within the gland, since it was also observed during stimulation by CCK-Pz ($2{\cdot}5 \times 10^{-2}$ Crick-Harper-Raper u./min) in the presence of atropine (10 mg/l.) in three experiments.

The effects of barium on pancreatic secretion. In two experiments barium (2·5–5·0 m-equiv/l.) did not prevent the reduction in electrolyte and amylase secretion associated with prolonged calcium-free perfusion or aid the recovery of electrolyte secretion after calcium depletion.

Alterations in perfusate [Mg]

The effect of magnesium-rich perfusate on pancreatic secretion. Perfusion with magnesium-rich fluids (10 m-equiv Mg/l.) for up to 60 min did not inhibit electrolyte secretion or acetylcholine-stimulated amylase secretion.

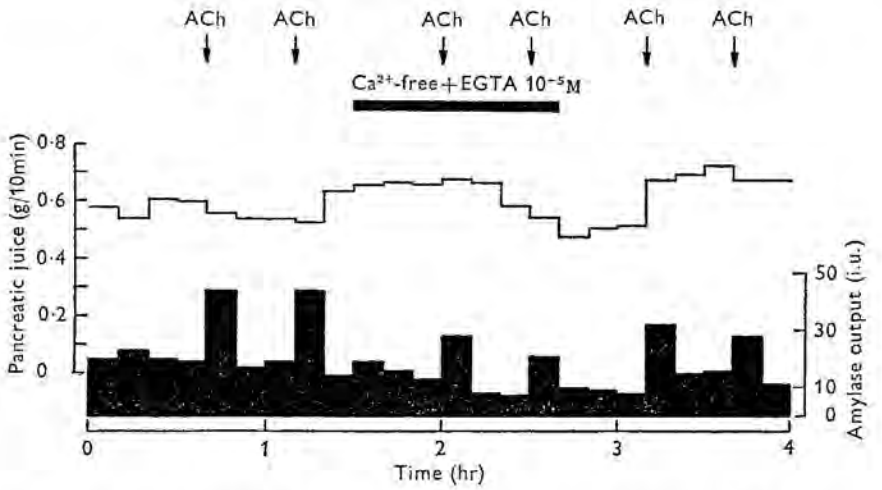

Fig. 6. The effect of calcium-free perfusate on amylase secretion from a perfused cat pancreas. Electrolyte secretion was maximally stimulated throughout by infusing secretin at a supramaximal dose (30 μg/min). A calcium-free solution containing EGTA (10^{-5} M) was perfused through the gland for the duration of the horizontal bar. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

In fact, like calcium, excess magnesium caused a transient increase in basal enzyme secretion and potentiated minimal stimulation by acetylcholine, but the effects were never as great as those observed with calcium.

The effects of magnesium-free perfusates on pancreatic secretion. Magnesium-free solutions had no effect on electrolyte secretion or on acetylcholine-stimulated enzyme secretion when perfused through the gland for up to 60 min (two experiments).

Alterations in perfusate [Na]

The effect of sodium-deficient perfusates on pancreatic secretion. Sodium deficiency is known to inhibit pancreatic electrolyte secretion (Case *et al.* 1968). Its effect on enzyme secretion was tested in five experiments by perfusion with solutions containing either 0 or 50 mM-Na/l. (Fig. 9). After 50 min perfusion with 50 mM-Na/l., the response to acetylcholine was

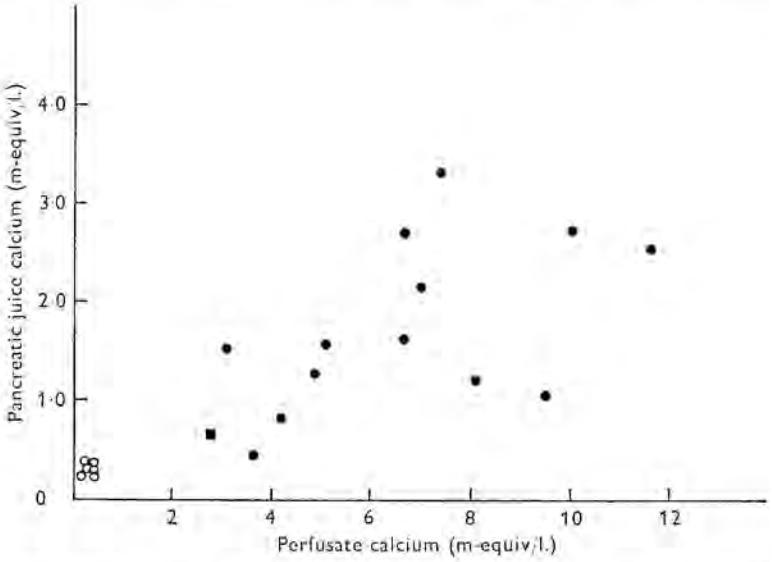

Fig. 7. The relationship between the concentration of calcium in perfusate and pancreatic juice. The closed circles represent single observations from a total of three experiments in which the concentration of calcium in the perfusion fluid was varied. The open circles represent single observations from six experiments in which the gland was perfused with a calcium-free solution containing EGTA. The filled square represents seventy-eight observations from eleven experiments in which the gland was perfused with the normal perfusion fluid.

normal though, because of the slow secretory rate, the enzyme was not all eliminated from the duct system during the 10 min test period, most of it appearing in the period following return to normal perfusate. A return to normal perfusate alone produced only a minimal increase in enzyme secretion, which presumably was a washing out of basal secretion that had accumulated in the ducts during the period of low electrolyte secretion. Similar observations were obtained with sodium-free solutions, though in these experiments electrolyte secretion often ceased completely.

Alterations in perfusate [K]

The effect of potassium-free solutions on pancreatic secretion. Potassium omission reduces pancreatic electrolyte secretion by about 60 % (Case, Harper & Scratcherd, 1969*b*). However, in three experiments, potassium-free fluids did not affect acetylcholine-stimulated amylase secretion (Fig. 10). In these experiments an increase in basal enzyme secretion was observed during potassium omission, an effect which was blocked by atropine.

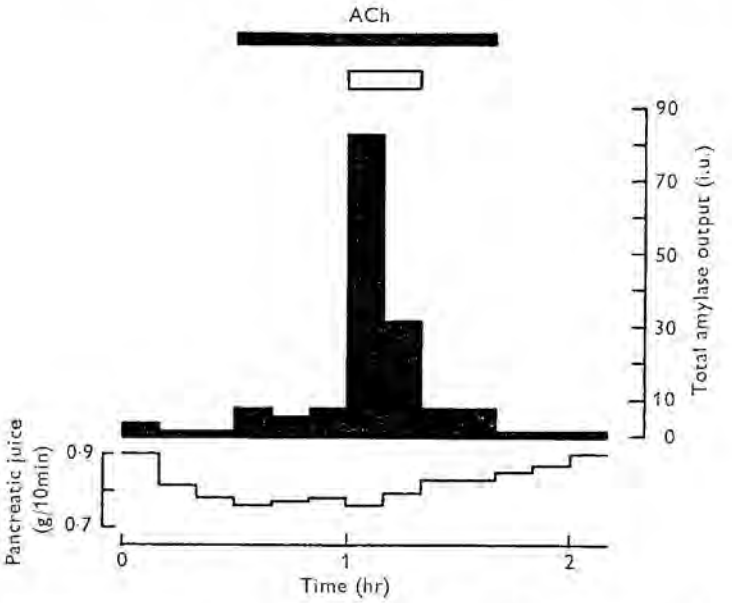

Fig. 8. The effect of increasing the perfusate calcium concentration during submaximal amylase secretion. Acetylcholine (5 ng/min) was infused into the gland for the period indicated by the filled bar. For the duration of the open bar the perfusate contained calcium at a concentration of 10 m-equiv/l. Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supramaximal dose (30 μg/min).

The effect of potassium-rich perfusates on pancreatic secretion. Solutions containing potassium at concentrations greater than 30 mM/l. cause acetylcholine release from nerve terminals within the pancreas and thus secondarily cause amylase secretion (Argent, Case & Scratcherd, 1971). This observation alone suggests that the enzyme secretory process of the acinar cell is unaffected by high extracellular potassium concentrations. In seeking support for this view, the effect of potassium-rich perfusion fluid on CCK-Pz-stimulated secretion was tested in three experiments. The

perfusion fluid contained atropine sulphate (10 mg/l.) to block the action of released acetylcholine. The response to CCK-Pz was unaffected by high extracellular potassium concentrations (Fig. 11).

DISCUSSION

Amylase secretion by the isolated, perfused cat pancreas is clearly very similar to that described previously for the gland *in vivo* (Case, Harper & Scratcherd, 1969*a*), consisting of a small, continuous basal secretion which

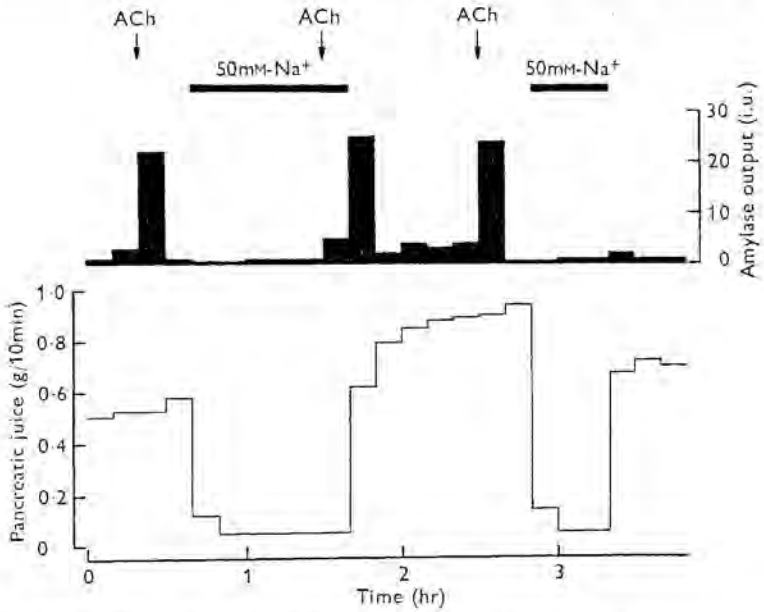

Fig. 9. The effects of sodium deficiency on electrolyte and amylase secretion from the perfused cat pancreas. Electrolyte secretion was stimulated maximally throughout by infusing crude secretin at a supramaximal dose (30 μg/min). For the duration of the horizontal bars the gland was perfused with a solution containing 50 mM-Na/l., isotonicity being maintained with sucrose. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

is supplemented in response to acetylcholine or CCK-Pz. As the perfusion fluids did not contain amino acids, the ability of the isolated gland to synthesize enzymes is limited by the size of the intracellular pool of amino acids, which accounts for the decreasing enzyme output in response to repeated large doses of enzyme stimulant. However, the consistent response to repeated small doses validates the use of this preparation in studying enzyme secretion.

The secretion of calcium in pancreatic juice. During maximal stimulation with secretin alone, the juice calcium concentration was about one quarter of that in the perfusate. Whenever the concentration of amylase in the secretion rose, either because the electrolyte secretory rate was very slow, or during transient or maintained stimulation with acetylcholine or CCK-Pz, the concentration of calcium rose in parallel. These observations confirm those made previously in the intact dog (Zimmerman, Dreilling, Rosenberg

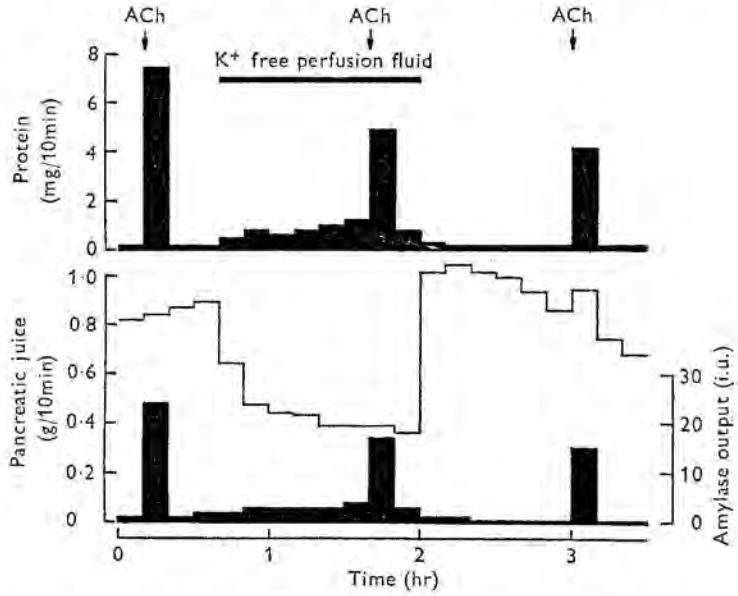

Fig. 10. The effects of potassium-free perfusion fluid on amylase, protein and electrolyte secretion from a perfused cat pancreas. Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supramaximal dose (30 μg/min). For the period denoted by the horizontal bar potassium was absent from the perfusion fluid. The arrows marked ACh indicate single injections of acetylcholine (200 ng).

& Janowitz, 1967; Zimmerman, Moore, Dreilling & Janowitz, 1971; Goebell, Steffan & Bode, 1972) and in man (Goebell, Bode & Horn, 1970). A similar close parallelism in the concentration of calcium and exportable protein exists in gastric (Moore & Makhlouf, 1968) and salivary (Dreisbach, 1967; Wallach & Schramm, 1971) secretions. Wallach & Schramm (1971) suggest that, in the parotid gland, calcium is packaged along with the exportable protein (calcium is known to form an internal chelate within the amylase molecule; Stein, Hsiu & Fischer, 1964; Hsiu, Fischer & Stein, 1964) and calcium may be similarly packaged in the pancreas, as zinc (Pekas, 1971) and inorganic sulphate (Berg & Young, 1971) are known to be.

However, not all calcium enters pancreatic juice bound to enzymes. Use of the saline-perfused pancreas allows the perfusate calcium concentration to be raised without the complication of binding to plasma proteins. Under such conditions the juice calcium concentration rose without a parallel increase in amylase secretion. Also, extrapolation of the regression line correlating the calcium and amylase outputs (Fig. 3) indicates that at zero

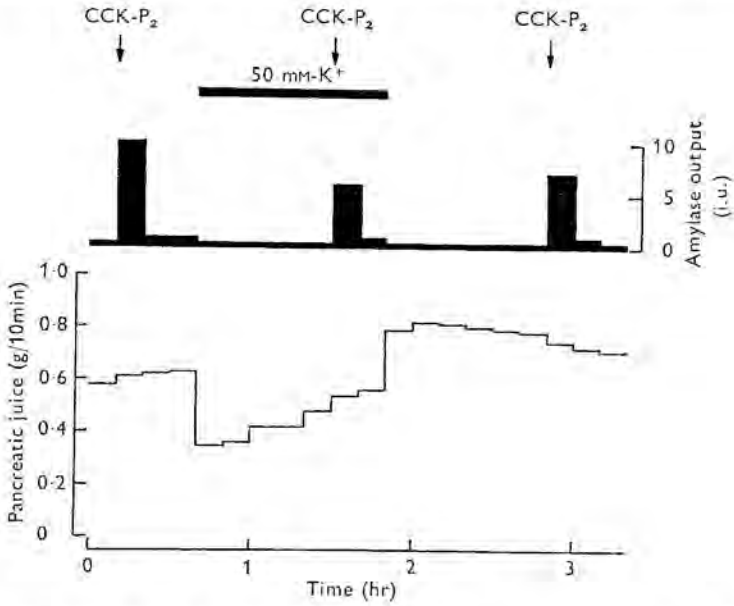

Fig. 11. The effect of excess potassium on amylase secretion from the perfused cat pancreas. For the duration of the horizontal bar the gland was perfused with a solution containing 50 mM-K. The arrows indicate single injections of pure CCK-Pz (1·0 Crick, Harper, Raper u.). To prevent the action of acetylcholine released by potassium from nerve terminal within the gland all perfusion fluids contained atropine (10 mg/l.). Electrolyte secretion was maximally stimulated throughout by infusing crude secretin at a supramaximal dose (30 μg/min).

amylase output pancreatic juice still contains calcium. This second calcium component must arise either by way of the electrolyte secretory mechanism or by diffusion through the duct system. A similar two-component hypothesis for pancreatic calcium secretion has been formulated independently by Goebell *et al.* (1972).

Effect of calcium and other cations on enzyme secretion. Potassium-rich fluids cause acetylcholine release from nerve terminals within the pancreas (Argent *et al.* 1971). The present experiments suggest that potassium-free fluids act in a similar way. Both procedures cause a secondary stimulation

of amylase secretion, due to the acetylcholine released, but do not directly stimulate the acinar cell to secrete enzymes, or influence the stimulatory effect of CCK-Pz or acetylcholine on these cells. Therefore the small depolarization of acinar cells due to acetylcholine and CCK-Pz (Dean & Matthews, 1972; Petersen & Matthews, 1972; J. R. Greenwell and T. Scratcherd, in preparation), although reflecting an altered membrane permeability, is presumably not responsible for enzyme secretion *per se*. Similar conclusions have been reached with regard to the actions of acetylcholine on the adrenal medulla (Douglas & Rubin, 1963; Douglas, Kanno & Sampson, 1967) and ACTH on the adrenal cortex (Matthews & Saffran, 1967; Jaanus, Rosenstein & Rubin, 1970).

Although the most likely cause of this small depolarization is an influx of external cation, removal of both major external cations (sodium and calcium) had little or no immediate effect on the secretory response to acetylcholine or CCK-Pz. Ridderstap & Bonting (1969) have also shown that basal enzyme secretion from isolated rabbit pancreas is unaffected during prolonged exposure to bathing fluid containing 25 m-mole Na/l. This point requires further clarification.

Although calcium removal had little immediate effect on amylase secretion, prolonged perfusion with calcium-free EGTA buffer did reduce basal secretion and almost abolish stimulated secretion. Inhibitory effects of calcium-free media on amylase secretion have been observed in other *in vitro* preparations of pancreas (Hokin, 1966; Robberecht & Christophe, 1971; Case & Clausen, 1971*a*, *b*) and parotid gland (Rasmussen & Tenenhouse, 1968; Selinger & Naim, 1970). However, inhibition was only partially reversible, suggesting that it may result from damage to the acinar cell.

In seeking evidence for a more direct role of extracellular calcium in enzyme secretion, the effects of tetracaine and of elevated extracellular calcium and magnesium concentrations were tested. The local anaesthetic tetracaine is known to inhibit catecholamine secretion from chromaffin cells by blocking the influx of calcium ions that occurs in response to acetylcholine stimulation (Douglas & Kanno, 1967). It certainly does inhibit amylase secretion at a concentration which is without effect on electrolyte secretion. However, tetracaine is also antagonistic to the action of acetylcholine (Rubin, Feinstein, Jaanus & Paimre, 1967), and this may explain its effect on the pancreas. The potentiating effects of excess calcium were rather small, and the inhibitory effects of excess magnesium non-existent; only weak inhibitory effects of magnesium have previously been described (Robberecht & Christophe, 1971) and there are no other reports of the effects of excess calcium.

The conclusion from this evidence is that extracellular calcium may not

play a very important direct role in enzyme secretion from the exocrine pancreas. This is in marked contrast to its established role in the secretion of transmitter from nerve endings and of hormones from endocrine cells where secretion appears to be triggered by calcium influx into the cell. In these tissues, secretion is directly related to extracellular calcium concentration and magnesium acts as an antagonist and barium as an agonist of calcium (see Rubin, 1970). Douglas (1968) has suggested that, in the secretion of macromolecules, calcium ions act as a coupling agent between the stimulus and the secretory mechanism. If this is the case in the pancreas the evidence above suggests that the source of the calcium ions may not be extracellular (as in neuro-endocrine tissue) but intracellular. This conclusion is supported by ^{45}Ca flux studies in rat pancreas, where acetylcholine and CCK-Pz have no effect on ^{45}Ca uptake, but do cause a dose-dependent acceleration of ^{45}Ca efflux (Case & Clausen, 1971*a*, *b*; R. M. Case and T. Clausen, in preparation). However, the rat pancreas responds differently to gastrointestinal hormones than the cat pancreas (Dockray, 1972) and it may be unwise to compare too closely data obtained in the two species. Intracellular calcium may also be used in the regulation of amylase secretion from salivary glands (Nielsen & Petersen, 1972). A more detailed consideration of these points is presented elsewhere (Case, 1973).

Effect of calcium on electrolyte secretion. The inhibitory effect of calcium-free media on pancreatic electrolyte secretion, which has not previously been described, was slower to develop than that on enzyme secretion. A similar situation exists in the submaxillary gland (Douglas & Poisner, 1963). This suggests a different sensitivity of the two secretory processes to calcium. This delayed inhibitory effect is in marked contrast to the immediate effects produced by removal of sodium, potassium or bicarbonate in pancreas (Case *et al.* 1968, 1969*b*; Case, Scratcherd & Wynne, 1970) or of sodium in the submaxillary gland (Martinez & Petersen, 1972).

A calcium requirement for gastric acid secretion has also been demonstrated (Forte & Nauss, 1963; Jacobson, Schwartz & Rehm, 1965). The cause of inhibition in these and other electrolyte transport processes is difficult to assess. It may result from alterations in the permeability of the cell membrane (Manery, 1966); or of the junctional complex between cells, which apparently acts as the principal route of passive ion permeation in gall bladder, and perhaps other tissues (Diamond, Barry & Wright, 1971). Certainly EDTA treatment causes increased movement of sucrose across bullfrog gastric mucosa (Forte & Nauss, 1963) by loosening the junctional complex between gastric cells (Sedar & Forte, 1964) and a similar explanation may account for the increased calcium concentration in pancreatic juice collected immediately after a prolonged period of calcium-free

perfusion. Whether calcium ions have a direct effect on the electrolyte secretory mechanism remains to be determined.

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REFERENCES

ARGENT, B. E., CASE, R. M., FRASER, M. P. & SCRATCHERD, T. (1972). The effects of calcium on volume and amylase secretion from the perfused cat pancreas. *J. Physiol.* **224**, 29–30*P*.

ARGENT, B. E., CASE, R. M. & SCRATCHERD, T. (1971). Stimulation of amylase secretion from the perfused cat pancreas by potassium and other alkali metal ions. *J. Physiol.* **216**, 611–624.

BERG, N. B. & YOUNG, R. W. (1971). Sulphate metabolism in pancreatic acinar cells. *J. cell Biol.* **50**, 469–483.

CASE, R. M. (1973). Calcium and gastrointestinal secretion. *Digestion* (in the Press).

CASE, R. M. & CLAUSEN, T. (1971*a*). Calcium ions and the release of enzymes from rat pancreas in response to gastrointestinal hormones. *Acta endocr. Copenh.* suppl. 155, 203.

CASE, R. M. & CLAUSEN, T. (1971*b*). Stimulus-secretion coupling in exocrine pancreas. II. The role of calcium ions. In *Alfred Benzon Publication I. Gastrointestinal Hormones and Other Subjects*, ed. THAYSEN, E. H., pp. 62–66. Copenhagen: Munksgaard.

CASE, R. M., HARPER, A. A. & SCRATCHERD, T. (1968). Water and electrolyte secretion by the perfused pancreas of the cat. *J. Physiol.* **196**, 133–149.

CASE, R. M., HARPER, A. A. & SCRATCHERD, T. (1969*a*). The secretion of electrolytes and enzymes by the pancreas of the anaesthetized cat. *J. Physiol.* **201**, 335–348.

CASE, R. M., HARPER, A. A. & SCRATCHERD, T. (1969*b*). Water and electrolyte secretion by the pancreas. In *The Exocrine Glands*, ed. BOTELHO, S. Y., BROOKS, F. P. & SHELLY, W. B., pp. 39–60. Philadelphia: University of Pennsylvania Press.

CASE, R. M., SCRATCHERD, T. & WYNNE, R. D'A. (1970). The origin and secretion of pancreatic juice bicarbonate. *J. Physiol.* **210**, 1–15.

CRICK, J., HARPER, A. A. & RAPER, H. S. (1949). On the preparation of secretin and pancreozymin. *J. Physiol.* **110**, 367–376.

DEAN, P. M. & MATTHEWS, E. K. (1972). Pancreatic acinar cells: measurement of membrane potential and miniature depolarization potentials. *J. Physiol.* **226**, 1–13.

DIAMOND, J. M., BARRY, P. & WRIGHT, E. M. (1971). The route of transepithelial ion permeation in the gall-bladder. In *Symposia Medica Hoechst, Electrophysiology of Epithelial Cells*, pp. 22–38. Stuttgart, New York: Schattauer Verlag.

DOCKRAY, G. J. (1972). The action of secretin, cholecystokinin-pancreozymin and caerulein on pancreatic secretion in the rat. *J. Physiol.* **225**, 679–692.

DOUGLAS, W. W. (1968). Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmac. Chemother.* **34**, 451–474.

DOUGLAS, W. W. & KANNO, T. (1967). The effect of amecothaine on acetylcholine-induced depolarization and catecholamine secretion in the adrenal chromaffin cell. *Br. J. Pharmac. Chemother.* **30**, 612–619.

DOUGLAS, W. W., KANNO, T. & SAMPSON, S. R. (1967). Influence of the ionic environment on the membrane potential of adrenal chromaffin cells and on the depolarizing effect of acetylcholine. *J. Physiol.* **191**, 107–121.

DOUGLAS, W. W. & POISNER, A. M. (1963). The influence of calcium on the secretory response of the submaxillary gland to acetylcholine or to noradrenaline. *J. Physiol.* **165**, 528–541.

DOUGLAS, W. W. & RUBIN, R. P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J. Physiol.* **167**, 288–310.

DREISBACH, R. H. (1967). Effect of isoproterenol on calcium, protein and electrolytes of rat submaxillary gland. *Proc. Soc. exp. Biol. Med.* **126**, 279–281.

FORTE, J. G. & NAUSS, A. H. (1963). Effects of calcium removal on bullfrog gastric mucosa. *Am. J. Physiol.* **205**, 631–637.

GOEBELL, H., BODE, C. & HORN, H. D. (1970). Einfluss von Sekretin und Pankreozymin auf die Calciumsekretion im menschlichen Duodenalsaft bei normaler und gestörter Pankreasfunktion. *Klin. Wschr.* **48**, 1330–1339.

GOEBELL, H., STEFFAN, C. & BODE, C. (1972). Stimulatory effect of pancreozymin-cholecystokinin on calcium secretion in pancreatic juice of dogs. *Gut* **13**, 477–482.

HOKIN, L. E. (1966). Effects of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. *Biochim. biophys. Acta* **115**, 219–221.

HSIU, J., FISCHER, E. H. & STEIN, E. A. (1964). Alpha-amylases as calcium-metalloenzymes. II. Calcium and the catalytic activity. *Biochemistry* **3**, 61–66.

JAANUS, S. D., ROSENSTEIN, M. J. & RUBIN, R. P. (1970). On the mode of action of ACTH on the isolated perfused adrenal gland. *J. Physiol.* **209**, 539–556.

JACOBSON, A., SCHWARTZ, M. & REHM, W. S. (1965). Effects of removal of calcium from bathing media on frog stomach. *Am. J. Physiol.* **209**, 134–140.

LAGERLÖF, H. O. (1942). *Pancreatic Function and Pancreatic Disease Studied by Means of Secretin.* Stockholm: Norsted and Söner.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MANERY, J. F. (1966). Effect of Ca ions on membranes. *Fedn Proc.* **25**, 1804–1810.

MARTINEZ, J. R. & PETERSEN, O. H. (1972). The importance of extracellular calcium for acetylcholine-evoked salivary secretion. *Experientia* **28**, 167–168.

MATTHEWS, E. K. & SAFFRAN, M. (1967). Steroid production and membrane potential measurements in cells of the adrenal cortex. *J. Physiol.* **189**, 149–161.

MOORE, E. W. & MAKHLOUF, G. M. (1968). Calcium in normal human gastric juice. A four-component model with speculation on the relation of calcium to pepsin secretion. *Gastroenterology* **55**, 465–480.

NIELSEN, S. & PETERSEN, O. H. (1972). Transport of calcium in the perfused submandibular gland of the cat. *J. Physiol.* **223**, 685–697.

PEKAS, J. C. (1971). Pancreatic incorporation of ^{65}Zn and histidine-^{14}C into secreted proteins of the pig. *Am. J. Physiol.* **220**, 799–803.

PETERSEN, O. H. & MATTHEWS, E. K. (1972). The effect of pancreozymin and acetylcholine on the membrane potential of the pancreatic acinar cells. *Experientia* **28**, 1037–1038.

RASMUSSEN, H. & TENENHOUSE, A. (1968). Cyclic adenosine monophosphate, Ca^{2+}, and membranes. *Proc. natn. Acad. Sci. U.S.A.* **59**, 1364–1370.

RIDDERSTAP, A. S. & BONTING, S. L. (1969). Enzyme secretion by the isolated rabbit pancreas: absence of a relation with the Na–K activated ATPase. *Pflügers Arch. ges. Physiol.* **313**, 53–61.

ROBBERECHT, P. & CHRISTOPHE, J. (1971). Secretion of hydrolases by perfused fragments of rat pancreas: effect of calcium. *Am. J. Physiol.* **220**, 911–917.

RUBIN, R. P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. *Pharmac. Rev.* **22**, 389–428.

RUBIN, R. P., FEINSTEIN, M. B., JAANUS, S. D. & PAIMRE, M. (1967). Inhibition of catecholamine secretion and calcium exchange in perfused cat adrenal glands by tetracaine and magnesium. *J. Pharmac. exp. ther.* **155**, 463–471.

SCHRAMM, M. (1967). Secretion of enzymes and other macromolecules. *A. Rev. Biochem.* **36**, 307–320.

SEDAR, A. W. & FORTE, J. G. (1964). Effects of calcium depletion on the junctional complex between oxyntic cells of gastric glands. *J. cell Biol.* **22**, 173–188.

SELINGER, Z. & NAIM, E. (1970). The effect of calcium on amylase secretion by rat parotid slices. *Biochim. biophys. Acta* **203**, 335–337.

STEIN, E. A., HSIU, J. & FISCHER, E. H. (1964). Alpha amylases as calcium metalloenzymes. I. Preparation of calcium-free apoamylases by chelation and electrodialysis. *Biochemistry* **3**, 56–61.

WALLACH, D. & SCHRAMM, M. (1971). Calcium and the exportable protein in rat parotid gland. Parallel subcellular distribution and concomitant secretion. *Eur. J. Biochem.* **21**, 433–437.

ZIMMERMAN, M. J., DREILLING, D. A., ROSENBERG, I. R. & JANOWITZ, H. D. (1967). Secretion of calcium by the canine pancreas. *Gastroenterology* **52**, 865–870.

ZIMMERMAN, M. J., MOORE, E. W., DREILLING, D. A. & JANOWITZ, H. D. (1971). Pancreatic juice ionized and total calcium in dogs in response to secretin, pancreozymin and calcium infusions. *J. clin. Invest.* **50**, 103*a*.